Possibilities of mucosal protection in bile-induced esophageal inflammation

Ph.D. Thesis

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II. Szentpáli Károly, Kaszaki József, Erős Gábor, Tiszlavicz László, Paszt Attila, Lázár György, Balogh Ádám, Boros Mihály: A nyelőcső nyálkahártya mikrokeringési változásai kísérletes reflux alatt. Az epés komponens kóroki szerepe. Magyar Sebészet 2003; 56: 61-67

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IV. Gábor Erős, József Kaszaki, Miklós Czóbel, Mihály Boros: Systemic phosphatidylcholine pretreatment protects canine esophageal mucosa during acute experimental biliary reflux. World J Gastroenterol 2006; 12(2):271-279

LIST OF ABSTRACTS RELATED TO THE SUBJECT OF THE DISSERTATION

1. Szentpáli K, Erős G, Kaszaki J, Tiszlavicz L, Lázár Gy, Balogh Á, Boros M: *In vivo* observation of microcirculatory changes during experimental gastro-esophageal reflux. Walter Brendel Presentation. Eur Surg Res 33: 107, 2001.
2. Erős G, Kaszaki J, Szentpáli K, Boros M: A nyelőcső nyálkahártya mikrokeringése kísérletes gastro-oesophagealis reflux alatt. Magyar Sebészet Suppl. p 47, 2001.
3. Erős G, Kaszaki J, Szentpáli K, Boros M: The role of nitric oxide in the pathomechanism of biliary gastro-esophageal reflux. Eur Surg Res 34 (S1):7, 2002
4. Erős G, Kaszaki J, Szentpáli K, Boros M: The role of nitric oxide in the pathomechanism of bile-induced esophagitis. Shock 18 (S1): 6, 2002.
5. Erős G, Kaszaki J, Ghyczy M, Boros M: Systemic phosphatidylcholine pre-treatment protects the esophageal tissue during acute experimental biliary reflux. Walter Brendel Presentation. Eur Surg Res 35: 183, 2003.
6. Erős G, Kaszaki J, Boros M: A hízósejtek szerepe a a nyelőcső nyálkahártya mikrokeringési változásaiban epés reflux alatt. A foszfatidilkolin preventív hatása. Érbetegségek (S1): 10, 2005.

1. LIST OF ABBREVIATIONS

ATP:	Adenosine triphosphate
DMSO:	Dimethyl sulfoxide
EDTA:	Ethylenediaminetetraacetic acid
EGTA:	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FCD:	Functional capillary density
GERD:	Gastroesophageal reflux disease
HCl:	Hydrochloric acid
H&E:	Hematoxylin-eosin
LES:	Lower esophageal sphincter
MAP:	Mean arterial pressure
MC:	Mast cell
MPO:	Myeloperoxidase
NADPH:	Nicotinamide adenine dinucleotide phosphate
NF:	Nuclear factor
7-NI:	7-Nitroindazole
NO:	Nitric oxide
NOS:	Nitric oxide synthase
cNOS:	Constitutively expressed nitric oxide synthase
eNOS:	Endothelial nitric oxide synthase
iNOS:	Inducible nitric oxide synthase
nNOS:	Neuronal nitric oxide synthase
PC:	Phosphatidylcholine
PMNG:	Polymorphonuclear leukocyte
PPI:	Proton pump inhibitor
RBCV:	Red blood cell velocity
RVA:	Relative vessel area
VD:	Vessel diameter

2. INTRODUCTION

2.1. BACKGROUND

Regurgitation of the gastric or duodenal content into the esophageal lumen is virtually an everyday phenomenon in everyone. Nevertheless, repeated regurgitation, together with the insufficiency of antireflux mechanisms, may lead to gastro-esophageal reflux disease (GERD), one of the most common gastrointestinal diseases in Europe. Recent epidemiological studies revealed that 10-48% of the population suffer from GERD symptoms, and the prevalence of GERD is likewise increasing in other parts of the world (**Goh *et al.*, 2004, Fujiwara *et al.*, 2005**).

The leading symptoms of the disease are heartburn, noncardiac chest pain, dysphagia or odynophagia. Extraesophageal symptoms include chronic cough, laryngitis, asthma, chronic sinusitis and dental erosions.

The etiology of GERD is clearly multifactorial. Incompetency of the lower esophageal sphincter (LES), a decreased esophageal clearance, a weak esophagus body motility, abnormal gastric emptying and hiatal hernia are important factors. An increased intraabdominal pressure (*e.g.* obesity, obstipation, ascites or pregnancy) and medication which weakens the LES function (*e.g.* anticholinergic drugs or calcium-channel blocking agents) can additionally contribute to the pathogenesis of GERD.

Prolonged regurgitation of the stomach or duodenal content can lead to different complications such as esophagitis, ulcer, stricture and Barret's esophagus, which predisposes to malignancy. Barret's esophagus is a metaplastic condition that affects the lower esophagus, where the normal squamous epithelium is replaced by columnar lined epithelium. This complication occurs in 15 to 20% of GERD patients (**Hirota *et al.*, 1999, DeMeester *et al.*, 2000**). Indeed, the prevalence of adenocarcinoma of the esophagus and esophagogastric junction is increasing at an extraordinary rate (**Devesa *et al.*, 1998**).

Gastric acid has been shown to play a crucial role in the development of esophagitis and it was traditionally considered to be the main pathogenetic factor of GERD. Consequently, the cornerstone of therapy was virtually complete acid suppression through use of proton pump inhibiors (PPIs). However, PPI treatment has proved to be ineffective in several cases and esophagitis is a frequent finding even after total gastrectomy (**Bremner *et al.*, 1993**). In recent decades, the association between esophageal inflammation and the role of regurgitated bile were extensively studied (**Attwood *et al.*, 1989, Miwa *et al.*, 1996, Vaezi *et***

***al.*, 1996**). Bile can often be detected in the esophagus, and is assumed to trigger the development of Barret's metaplasia and esophageal adenocarcinoma. With appropriate diagnostic tools (esophageal endoscopy, 24-h pH-metry or Bilitec examination), the presence of bile in the esophageal lumen has been proved in many cases. Accordingly, biliary or alkaline reflux has become a well-known and widely accepted entity in clinical practice. Further studies concluded that the mixed reflux of gastric and duodenal juices is more harmful than that of gastric acid alone (**Kauer *et al.*, 1995**).

Although GERD is a chronic disease, critically ill or respirated patients may encounter an acute acidic or biliary challenge. However, regardless of the time factor, the treatment of alkaline esophagitis can be difficult. PPIs inhibit both gastric acidity and the secreted volume, but these compounds target only the gastric secretion. The efficacy of bile acid-binding agents is still unproven. In patients with medically refractory symptoms, conventional and minimally invasive surgical techniques can reduce the morbidity (**Richter, 2004**). Nevertheless, a non invasive, effective medical treatment for alkaline reflux is still not in use to date. A major obstacle could be that appropriate treatment or prevention of this condition requires an understanding of the pathogenesis of reflux-induced lesions and the nature of the inflammatory complications.

Although recent experimental and clinical studies have clarified many details of the pathogenesis of GERD, the exact pathophysiology of the reflux-induced mucosal dysfunction leading to clinicopathologic complications is still unknown. As compared with an acidic reflux, much less information has been obtained on bile-induced inflammation. It has been shown that regurgitated gastric acid leads to vasodilation and increases the mucosal blood flow. This vasodilation is mainly due to the effect of paracrine nitric oxide (NO). Moreover, the histamine released from activated mast cells (MCs) increases the mucosal blood flow via a NO-dependent mechanism, which is considered to be an important protective reaction against intraluminal noxious agents (**Sandler *et al.*, 1993, Feldman *et al.*, 1996**).

It has been demonstrated that bile-induced mucosal changes *per se* may contribute significantly to esophageal barrier lesions and the development of GERD (**Bremner *et al.*, 1993**). Bile acids are responsible for the harmful effects of bile. Their cytotoxicity was first revealed in hepatocytes and intestinal epithelial cells. Earlier studies indicated that bile also plays an important role in the pathogenesis of gastritis, duodenitis and peptic ulcer. Bile acids may damage the mucosa in many ways. At above the critical micellar concentration in the extracellular space they act as a detergent and attack the lipid component of the membranes. Bile acids are able to enter cells due to their lipophilicity and to interfere with different cell

functions. Several lines of indirect evidence suggest that a mitochondrial dysfunction may play a role in the reflux-induced esophageal mucosal responses. The hepatic mitochondria are one of the main targets of bile-induced hepatocyte damage, and the intracellular accumulation of hydrophobic bile salts during cholestasis causes hepatocyte necrosis by inducing a mitochondrial permeability transition (**Spievy *et al.*, 1993, Gores *et al.*, 1998**). It has been demonstrated that bile action leads to adenosine triphosphate (ATP) depletion and mucosal damage during reflux esophagitis (**Szentpáli *et al.*, 2001**).

Attention should be paid to the role of NO in the pathophysiology of GERD. NO is a significant regulator of the mucosal blood flow in the gastrointestinal tract. The production of NO is linked to different isoforms of the enzyme nitric oxide synthase (NOS) (**McKie *et al.*, 1995, Salzman 1995**). The NOS system consists of three distinct isoforms: neuronal NOS (nNOS), inducable NOS (iNOS) and endothelial NOS (eNOS). Both eNOS and nNOS are constitutively expressed (cNOS), but nNOS is predominant in the gastrointestinal tract (**Fischer *et al.*, 1999, Qu *et al.*, 1999**). A relative lack of NO can activate both MCs and leukocytes, and MC degranulation *per se* can bring about leukocyte accumulation and other characteristics of local inflammation (**Kanwar *et al.*, 1994, Boros 2003**). These data suggest that nNOS-derived NO can be a significant factor of mucosal homeostasis.

Although mixed or biliary reflux is an important health care problem, to date there are no effective pharmacological therapies for bile-induced esophagitis. Our previous studies and various other lines of evidence have suggested that phosphatidylcholine (PC) may be protective during this condition. Stress induces the phospolipase D-catalyzed hydrolysis of membrane PC. This reaction leads to the endogenous production of phosphatidic acid and choline. Choline is a potent anti-inflammatory agent and is actively transported into the epithelial cells (**Kuehl *et al.*, 1957**). Choline could form part of a defense mechanism which may operate in biological systems against oxido-reductive stress (**Ghyczy *et al.*, 2003**). Furthermore, PC is taken up by phagocytic cells and accumulates in inflamed tissue (**Cleland *et al.*, 1979**). *In vitro* studies have demonstrated that PC may protect against the membrane damage caused by bile salts (**Martin *et al.*, 1981, el-Hariri *et al.*, 1992**). Further, *in vivo* studies have revealed that choline is an essential nutrient for humans and a choline deficiency may result in hepatic steatosis (**Zeisel *et al.*, 1991, Buchman *et al.*, 1995**). PC provides protection against many chemical toxin-induced pathological conditions, and especially liver damage (**Kidd, 1996**).

These data drew our attention to the possible importance of PC in bile-induced esophagitis and served as a guideline during design of our experiments.

2.2. AIMS

Our first goal was to design a large animal model of acute esophagitis in order to study the reactions of the esophageal mucosa exposed to different components of the refluxed material. Secondly, we set out to characterize the earliest indicators of inflammatory processes of the esophageal mucosa. With this aim, we observed mucosal microcirculatory changes by intravital videomicroscopy, and determined biochemical and histological markers to obtain an insight into the functional and structural changes accompanying reflux-induced inflammation. In this regard, the protocol was divided into two parts. In the first study, the major aims were:

- to create an appropriate animal model for the examination of acute esophagitis;
- to investigate the initial processes of acute mucosal destruction;
- to observe and characterize the intramural changes in the mucosal microcirculation;
- to study the roles of NO and the different NOS isoforms in acute esophagitis;
- to study the role of MCs in the pathogenesis of reflux-induced inflammation; and
- to compare the effects of regurgitated gastric acid and bile.

In addition to this investigation of the pathomechanism, another objective was to outline a means of modulating the outcome of biliary mucosal irritation. Accordingly, in a second study we set out to establish whether systemic PC administration can protect the esophageal mucosa by acting as an anti-inflammatory agent in bile-induced esophagitis. Here, the major aim was:

- to examine the protective role of systemic PC pretreatment during acute experimental gastroesophageal reflux.

3. MATERIALS AND METHODS

The experiments were performed in adherence to the NIH guidelines for the use of experimental animals. The study was approved by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged.

3.1. EXPERIMENTAL PROTOCOL

The experiments were performed on inbred mongrel dogs from the Animal House of the University of Szeged (average weight 12±3 kg) under sodium pentobarbital anesthesia (30 mg kg^{-1} i.v.). Small supplementary doses of pentobarbital were administered when necessary. Surgery was followed by a 30-min recovery period for cardiovascular stabilization. In the first study, 7 ml of isotonic saline (pH 7.4) was injected into the esophageal segment for 30 min in order to determine the baseline variables, including the microcirculatory parameters. After this period, the esophagus was filled with different test solutions (7 ml) for 3 h, while the microcirculatory parameters were continuously observed. At the end of the experiments, full-thickness biopsies were taken from the esophagus for histology hematoxylin-eosin (H&E) staining, NOS and myeloperoxidase (MPO) activity measurements. Additional biopsies were obtained with the freeze-clamp technique for determination of the tissue ATP content.

Study I: The animals were randomly allocated into one or other of the following groups. Group 1 (n=5) served as saline-treated control. Group 2 (n=8) received canine bile (pH 6.5). In group 3 (n=5), the animals were treated with hydrochloric acid (HCl) (pH 2.0). Group 4 (n=5) received bile + HCl solution (pH 2.5).

Study II: Cardiovascular stabilization was followed by systemic pretreatment protocols and 30 min later the baseline variables were determined. Thereafter, identical observations were performed and biopsies were taken as described above. In this study the sections were also stained with alcian blue-safranine O in order to visualize MCs. Animals were allocated into the following groups. Group 1 (n=5) was the saline-treated control group. Group 2 (n=8) received canine bile (pH 6.5). Group 3 (n=5) was pretreated with 5 mg kg^{-1} 7-nitroindazole (7-NI, Sigma Chem., USA) in a 0.3 ml min^{-1} iv infusion 30 min before bile administration. 7-NI is an *in vivo* selective inhibitor of nNOS. In group 4 (n=5), the animals received 50 mg kg^{-1} iv PC infusion 30 min before bile treatment. A 5% PC solution (soybean lecithin, MW: 785, Phospholipon 90, Phospholipid GmbH, Cologne, Germany) was freshly prepared according to the description of the manufacturer. Briefly, the PC solution contained deoxycholate (2.3%), NaOH (0.24%), benzyl alcohol (0.82%) and ethanol (0.27%) in distilled water. The intraluminal volume was identical in all groups studied.

3.2. SURGICAL PREPARATION

The surgical interventions in the first and second studies were identical (**Figure 1**). The animals were placed in a supine position on a heating pad for the maintenance of body temperature between 37 and 38 °C. In aseptic techniques, the left femoral artery and vein were cannulated for the measurement of mean arterial pressure (MAP) and for fluid and drug administration, respectively. All animals received a continuous infusion of Ringer's lactate at a rate of 10 ml kg^{-1} h^{-1} during the experiments. The esophagus was inspected by endoscopy (Olympus GIF-E) prior to the experiments to exclude major mucosal lesions. Following a collar incision, a part of the cervical esophagus with intact neurovascular connections was dissected free, and an approximately 8-10-cm segment of the middle portion was then occluded at both ends with atraumatic clips. A plastic tube (0.5 mm i.d.) was inserted distally into the lumen and secured with a purse-string suture for the administration of test compounds. The objective of the videomicroscope (Cytoscan A/R, Cytometrics, PA., USA) was introduced into the middle portion of the prepared esophageal segment through a small incision.

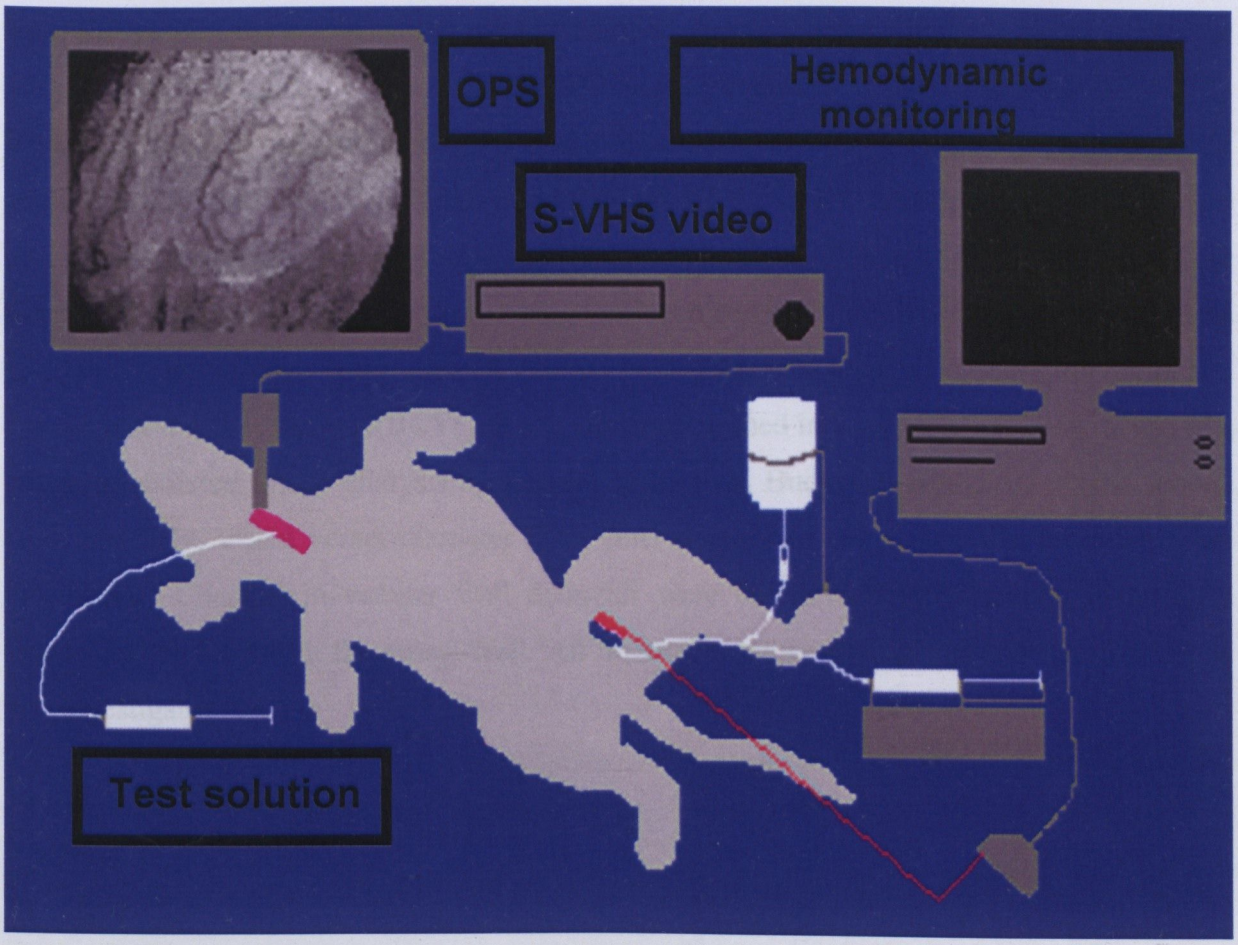

Figure 1. Experimental setup

3.3. MEASUREMENTS

Central venous pressure and MAP were measured continuously with Statham P23Db transducers and registered with a computerized data-acquisition system (Haemosys 1.17, Experimetria Ltd., Budapest, Hungary). Arterial blood gases were measured with an AVL Compact 2 blood gas analyzer (AVL Compact 2, Graz, Austria).

3 3.1. INTRAVITAL VIDEOMICROSCOPY

An intravital videomicroscope was used with an orthogonal polarization spectral imaging technique (Cytoscan A/R, Cytometrics, PA, USA) to monitor microcirculatory changes in the esophageal mucosa (**Figure 2**). This technique utilizes reflected polarized light at the wavelength of the isosbestic point of oxy- and deoxyhemoglobin (548 nm). Since polarization is preserved in reflection, only photons scattered from relatively deep inside the tissue contribute to the image formation. In this way, a virtual light source is created in the tissues, so that the vessels appear black (**Groner *et al.*, 1999**). A 10x objective was introduced into the esophageal lumen and was connected to a light source with a flexible cable; in this way, the esophageal segment was not exteriorized during the experiments. The microscopic images were recorded with an SVHS video recorder (Panasonic AG-TL 700). Videomicroscopic observations were made at specific anatomic locations at a depth of approximately 200 μm. Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images. In the first study, the functional capillary density (FCD; the length of perfused nutritive capillaries per observation area; μm^{-1}), the relative vessel area (RVA; the perfused vessel area per observation area), and the red blood cell velocity (RBCV; $\mu m\ s^{-1}$) were determined in 3 separate fields by means of a computer-assisted image analysis system (IVM Pictron, Budapest, Hungary). In the second study, the RBCV and vessel diameter (VD, μm) changes in the postcapillary venules were determined. Changes in venular wall diameter were determined by following identifiable visual landmarks within the vessel wall. All microcirculatory evaluations were performed by one investigator (GE).

Figure 2. The Cytoscan A/R videomicroscope (Cytometrics, USA)

3.3.2. NO SYNTHASE ACTIVITY MEASUREMENTS

NO formation in esophageal tissue was measured by the conversion of [^{3}H]L-citrulline from [^{3}H]L-arginine according to the method of **Szabó *et al.* (1993)**. Briefly, tissue biopsies kept on ice were homogenized in phosphate buffer (pH 7.4) containing 50 mM tris-(hydroxymethyl) aminomethane-HCl (Reanal, Budapest, Hungary), 0.1 mM ethylenediaminetetraacetic acid (Serva Feinbiochemica GmbH, Heidelberg, Germany), 0.5 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride and 10 μg ml^{-1} soybean trypsin inhibitor. The homogenate was centrifuged at 4 °C for 20 min at 24 000 g and the supernatant was loaded into centrifugal concentrator tubes (Amicon Centricon-100; 100 000 MW cut-off ultrafilter). The tubes were centrifuged at 1000 g for 150 min and the concentrated supernatant was washed out from the ultrafilter with 300 μl of homogenizing buffer. The samples were incubated with cation-exchange resin (DOWEX AG 500W-X8, Na^+ form) for 5 min to deplete endogenous L-arginine. The resin was separated by centrifugation (1500 g for 10 min) and the supernatant containing the enzyme was assayed for NOS activity.

For the Ca^{2+}-dependent NOS (cNOS) activity, 50 μl of enzyme extract and 100 μl of reaction mixture (pH 7.4, containing 50 mM Tris-HCl buffer, 1 mM beta-nicotinamide adenine dinucleotide phosphate (NADPH), 10 µM tetrahydrobiopterin, 1.5 mM $CaCl_2$, 100 U ml^{-1} calmodulin and 0.5 µCi [^{3}H]L-arginine (ICN Biomedicals, specific activity 39 Ci mmol^{-1}) were incubated together for 30 min at 37 °C. The reaction was stopped by the addition of 1 ml of ice-cold HEPES buffer (pH 5.5) containing 2 mM EGTA and 2 mM EDTA. Measurements were performed with boiled enzyme and with the NOS inhibitor N-ω-nitro-L-

arginine (3.2 mM) to determine the extent of [^{3}H]L-citrulline formation independent of the NOS activity. Ca^{2+}-independent NOS activity (iNOS) was measured without Ca-calmodulin and with EGTA (8 mM).

1 ml reaction mixture was applied to DOWEX cation-exchange resin (AG 50WX8, Na^+ form) and eluted with 2 ml of distilled water. The eluted [^{3}H]L-citrulline activity was measured with a scintillation counter (Tri-Carb Liquid Scintillation Analyzer 2100TR/2300TR, Packard Instrument Co, Meriden, CT, U.S.A.). Protein contents of samples were determined by the method of **Lowry *et al.* (1951)**.

3.3.3. MYELOPEROXIDASE ACTIVITY

The activity of MPO, a marker of tissue leukocyte infiltration, was measured in mucosal biopsies by the method of **Kuebler *et al.* (1996)**. Briefly, the tissue was homogenized with Tris-HCl buffer (0.1 M, pH 7.4) containing 0.1 mM polymethylsulfonyl fluoride to block tissue proteases, and then centrifuged at 4 °C for 20 min at 2000 g. During the measurements, 0.15 ml of 3,3',5,5'- tetramethylbenzidine (dissolved in DMSO; 1.6 mM) and 0.75 ml of hydrogen peroxide (dissolved in K_3PO_4 buffer; 0.6 mM) were added to 0.1 ml samples. The reaction causes the hydrogen peroxide-dependent oxidation of tetramethylbenzidine, which can be detected spectrophotometrically at 450 nm (UV-1601 spectrophotometer, Shimadzu, Japan). The MPO activities of the samples were measured at 37 °C; the reaction was stopped after 5 min with 0.2 ml of H_2SO_4 (2 M) and the data were referred to the protein content.

3.3.4. ATP MEASUREMENT

A whole-thickness sample was taken from the esophagus with a Wollenberg forceps cooled in liquid nitrogen, and the tissue was stored at -70 °C. The sample was weighed, placed into a 3-fold volume of trichloracetic acid (6% w/v), homogenized for 1 min, and centrifuged at 5000 g. The supernatant was neutralized with saturated potassium carbonate solution. The ATP concentration was measured spectrophotometrically according to **Lamprech *et al.* (1976)**. The method is based on the principle that ATP is used up in an enzymatic reaction catalyzed by glucose-6-phosphate dehydrogenase and hexokinase.

3.3.5. HISTOLOGY AND LIGHT MICROSCOPY

Biopsies for light microscopy were obtained from the intact and the treated part of the esophagus of each animal. The samples were fixed in 10% phosphate-buffered formalin

solution for 24 h, embedded in paraffin, sectioned (6 μm) and stained with H&E. Histological analysis was performed in coded sections by one investigator. In the first part of the study, mucosal injury was graded on the 0-100 esophageal mucosal damage score of **Lanas *et al.* (1999)** with the following criteria: epithelial changes (epithelial splitting, erosion and ulceration): maximal score 40; inflammation (intraepithelial leukocytes and cellular hyperplasia): maximal score 40; vascular lesions (edema, congestion and hemorrhage): maximal score 20. In parallel, the degree of damage was evaluated with the 0-16 scoring system of **Geisinger *et al.* (1990)**: basal cell hyperplasia: maximal score 4; intraepithelial leukocytes: maximal score 4; subepithelial leukocytes: maximal score 4; presence of ulceration: maximal score 4.

In the second study, polymorphonuclear leukocytes (PMNGs) were counted in coded sections by two independent investigators. Five nonoverlapping fields were observed in each section, and in each field an average of 4 consecutive measurements were used to calculate the average PMN count with a semiquantitative scoring system. PMN counts were determined in the epithelium, submucosa, muscle layer and adventitia with the following criteria: grade 0, no PMNs in the given structure; grade 1, 1-10 PMNs; grade 2, 11-100 PMNs; grade 3, >100 PMNs.

For histology for MC reactions, esophageal biopsy samples were rapidly placed into ice-cold Carnoy's fixative, embedded in paraffin and sectioned. The sections were stained with acidic toluidine blue (pH 0.5) and alcian blue-safranine O (Sigma Chem. USA, pH 0.4). Positively stained MCs were quantified in 10 fields. The counting was performed in coded sections at an optical magnification of x 400 by two independent investigators. Loss of intracellular granules and stained material dispersed diffusely within the lamina propria were taken as evidence of MC degranulation. For each animal, 5 fields were chosen at random, and the numbers of degranulated and intact MCs were counted. All counts were pooled for each treatment and the percentage of degranulated MCs was calculated.

3.3.6. STATISTICAL ANALYSIS

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Nonparametric methods were used. Friedman repeated measures analysis of variance on ranks was applied within the groups. Time-dependent differences from the baseline were assessed by Dunn's method. Differences between groups were analyzed with Kruskal-Wallis one-way analysis of variance on ranks,

followed by Dunn's method for pairwise multiple comparison. In the Figures, median values and 75th and 25th percentiles are given. $p<0.05$ was considered statistically significant.

4. RESULTS

4.1. STUDY I

The baseline values of the macrohemodynamic variables did not differ significantly in the different groups and there were no significant hemodynamic changes as compared with the control values during the experimental period (data not shown). In the control group, saline administration did not significantly influence the histology scores of the mucosal morphology.

The baseline level of the capillary RBCV in the various groups ranged between 430 and 470 μm s^{-1}. In the control group, the RBCV in the capillaries of the esophagus did not change during the experiments. However, the RBCV was increased significantly after the 3-h exposure to the bile or HCl-containing test solutions and average values of 607, 730 and 620 μm s^{-1} were measured after bile, HCl and bile + HCl treatment, respectively (Figure 3).

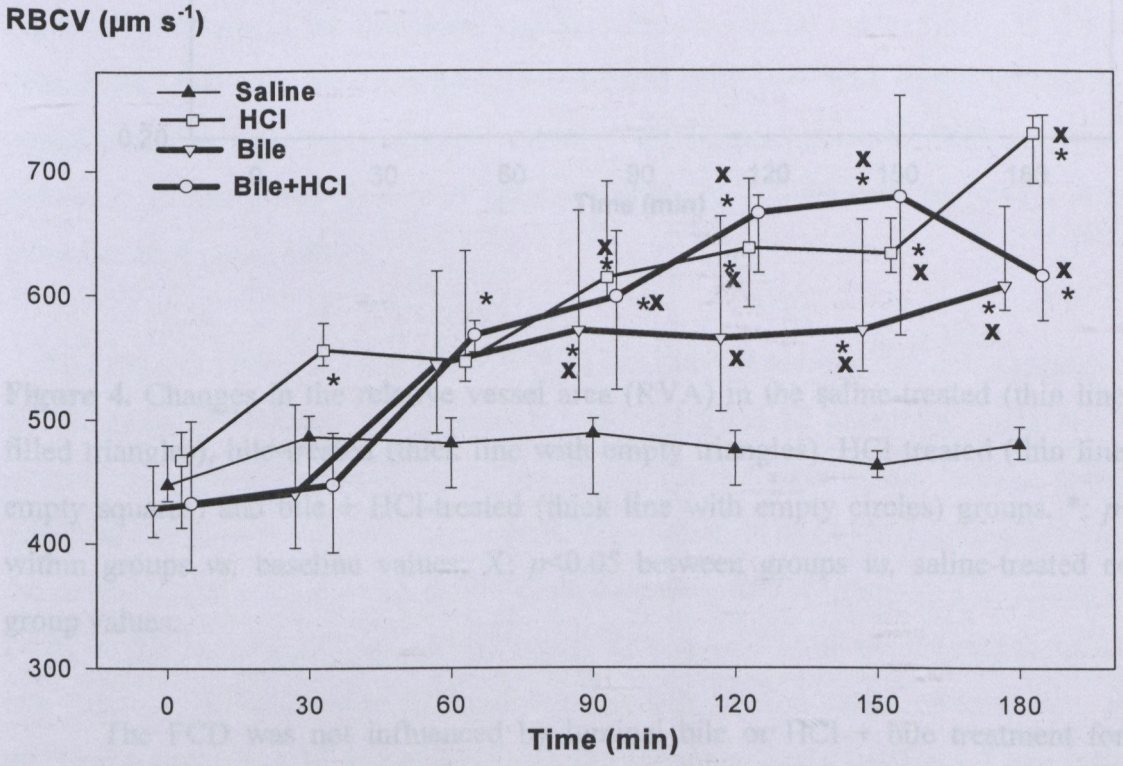

Figure 3. Effects of various intraluminar treatments on the red blood cell velocity (RBCV) of the esophageal mucosa (μm s^{-1}). Saline treatment (thin lines with filled triangles), bile

treatment (thick line with empty triangles), HCl treatment (thin line with empty squares) and bile + HCl treatment (thick line with empty circles). *: $p<0.05$ within groups *vs,* baseline values; X: $p<0.05$ between groups *vs,* saline-treated control group values.

The RVA was significantly elevated in all treated groups as compared with the sham-operated group or with the baseline. Administration of bile, HCl alone or bile + HCl resulted in a rise from the baseline values of 0.319, 0.333 and 0.312 to 0.437 (0.365; 0.473), 0.41 (0.391; 0.442) and 0.425 (0.415; 0.465), respectively (Figure 4).

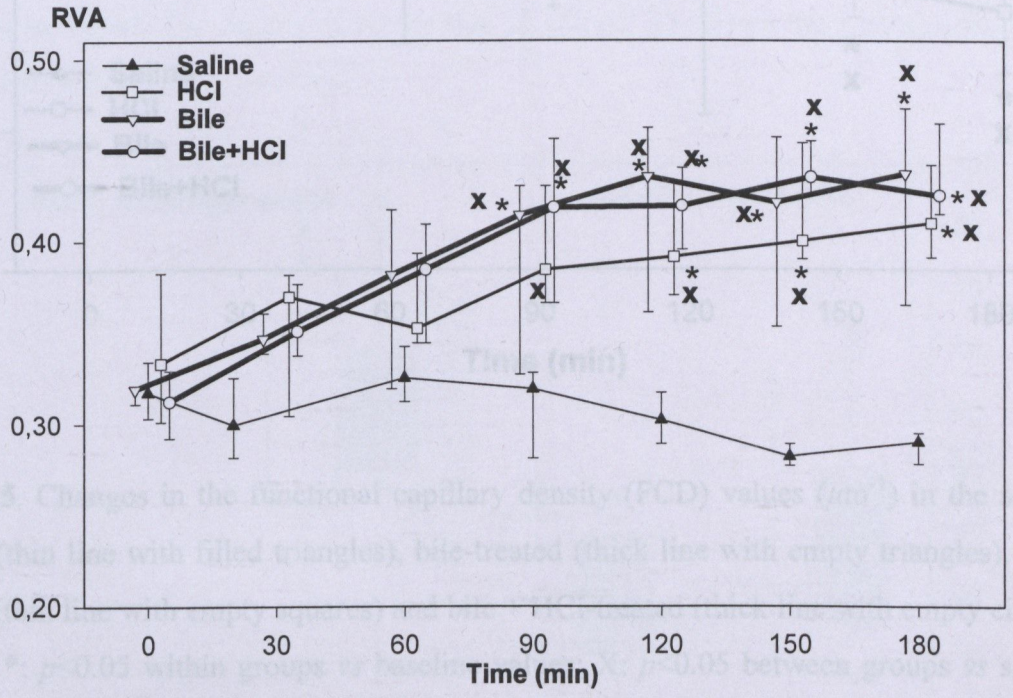

Figure 4. Changes in the relative vessel area (RVA) in the saline-treated (thin line with filled triangles), bile-treated (thick line with empty triangles), HCl treated (thin line with empty squares) and bile + HCl-treated (thick line with empty circles) groups. *: $p<0.05$ within groups *vs,* baseline values; X: $p<0.05$ between groups *vs,* saline-treated control group values.

The FCD was not influenced by luminal bile or HCl + bile treatment for 3 h. However, HCl treatment alone was followed by a significant decrease, from 0.0377 μm^{-1} (0.0342; 0.040) to 0.0292 μm^{-1} (0.027; 0.0307) at the end of the experimental period (Figure 5).

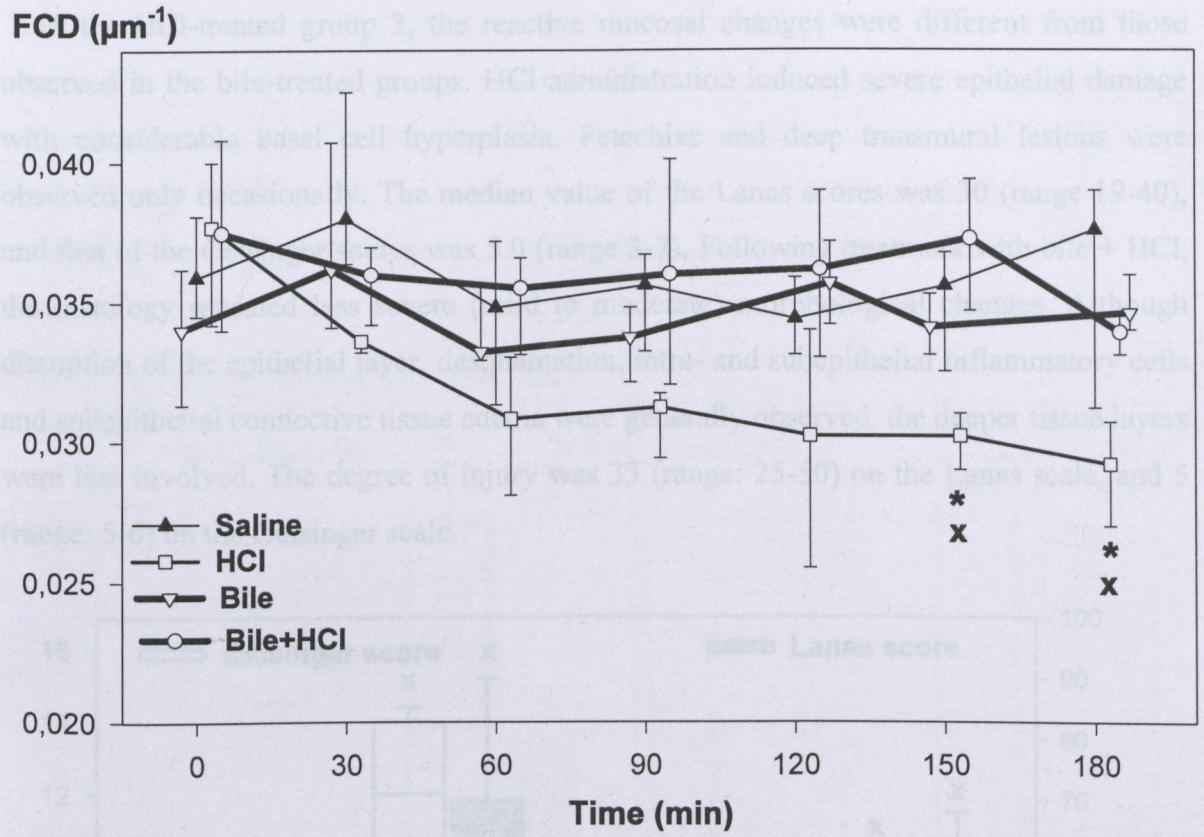

Figure 5. Changes in the functional capillary density (FCD) values (μm^{-1}) in the saline-treated (thin line with filled triangles), bile-treated (thick line with empty triangles), HCl-treated (thin line with empty squares) and bile + HCl-treated (thick line with empty circles) groups. *: $p<0.05$ within groups *vs* baseline values; X: $p<0.05$ between groups *vs* saline-treated control group values.

The effects of bile, HCl and bile + HCl on the mucosal morphology are presented in Figure 6. The structural damage to the mucosa was quantified by histology, using two different scoring systems; in consequence of the highly characteristic tissue lesions, there was no significant difference between the results of the evaluation methods. The biopsy samples from the saline-treated control group exhibited an average grade of injury of 2.25 (range of scores: 0-18) on the Lanas scale, and of 0.37 (range of scores: 0-3) on the Geisinger scale. The 3-h bile exposure induced severe mucosal damage, with median values of 65 (range: 50-70) on the Lanas scale, and 12 (range 8-14) on the Geisinger scale ($p<0.01$). Deep lesions were commonly observed, with extensive epithelial loss, desquamation and necrosis, intraepithelial and subepithelial leukocytosis, basal cell hyperplasia and subepithelial connective tissue damage. In most cases, transmural inflammation, edema and vasodilatation were apparent.

In the HCl-treated group 3, the reactive mucosal changes were different from those observed in the bile-treated groups. HCl administration induced severe epithelial damage with considerable basal cell hyperplasia. Petechiae and deep transmural lesions were observed only occasionally. The median value of the Lanas scores was 30 (range 19-40), and that of the Geisinger scores was 5.0 (range 3-7). Following treatment with bile + HCl, the histology revealed less severe (mild to moderate) morphological changes. Although disruption of the epithelial layer, desquamation, intra- and subepithelial inflammatory cells and subepithelial connective tissue edema were generally observed, the deeper tissue layers were less involved. The degree of injury was 33 (range: 25-50) on the Lanas scale, and 5 (range: 5-6) on the Geisinger scale.

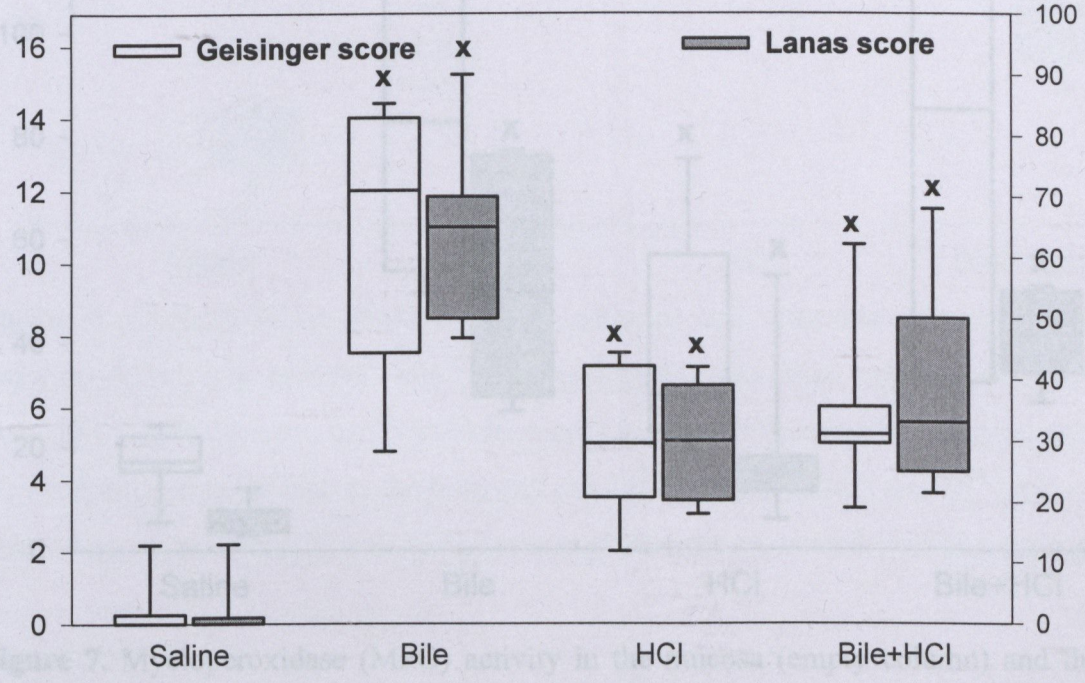

Figure 6. Scores of histological evaluation of the mucosal damage of the esophagus, with grading performed according to Lanas *et al.* and Geisinger *et al.* (see Methods for description). Geisinger score: empty column; Lanas score: shaded column. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles, respectively. X: $p<0.05$ between groups *vs,* saline-treated control group values.

The MPO data demonstrated that the leukocyte accumulation was significantly increased in the mucosa in groups 2, 3 and 4 as compared with the saline-treated group 1 (Figure 7). Bile, HCl alone or bile + HCl administration resulted in a 10-fold (M=50.0; 25p=29.5;

75*p*=76.1), 3-fold (*M*=15.0; 25*p*=11.2; 75*p*=17.9) and 8-fold (*M*=41.5; 25*p*=34.3; 75*p*=49.9) rise in MPO activity, respectively. Although the average MPO values were usually higher within the muscle, the MPO activities in the two tissue layers were not significantly different. Both bile and bile + HCl administration resulted in a 5-fold elevation, while HCl administration alone resulted in an approximately 2-fold increase in muscle MPO activity (Figure 7).

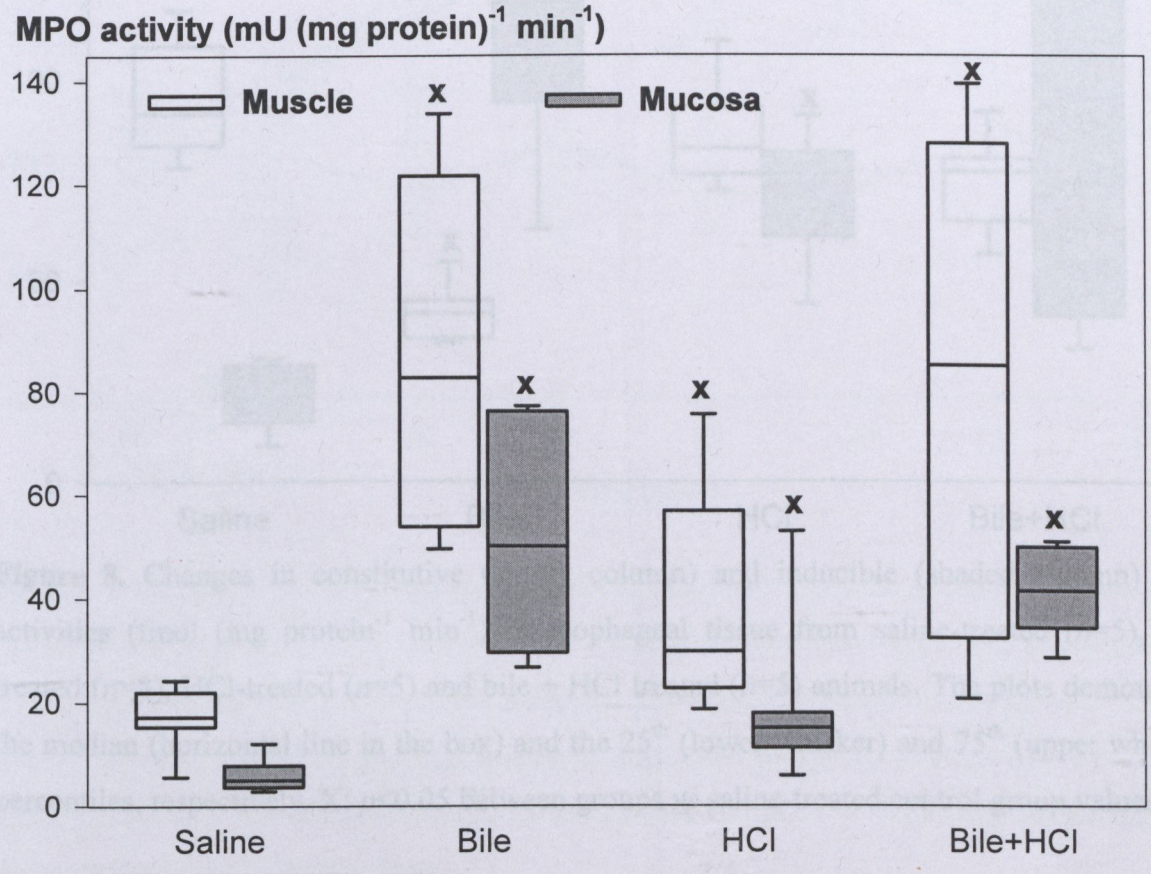

Figure 7. Myeloperoxidase (MPO) activity in the mucosa (empty column) and the muscle tissue (shaded column) of the esophagus 180 min after treatment. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles, respectively. X: $p<0.05$ between groups *vs* saline-treated control group values.

Figure 8 depicts the changes in the esophageal cNOS and iNOS activities. The activity of cNOS was significantly depressed after bile treatment, and this change was accompanied by a significant, approximately 6-fold increase in iNOS activity. HCl treatment did not influence the esophageal cNOS activity, while it resulted in a somewhat lower, 2.5-fold increase in iNOS activity as compared with the value for the sham-operated group. Similarly, the cNOS activity was not influenced by bile + HCl administration for 3 h, whereas the activity of iNOS was increased significantly, similarly as observed after bile treatment alone.

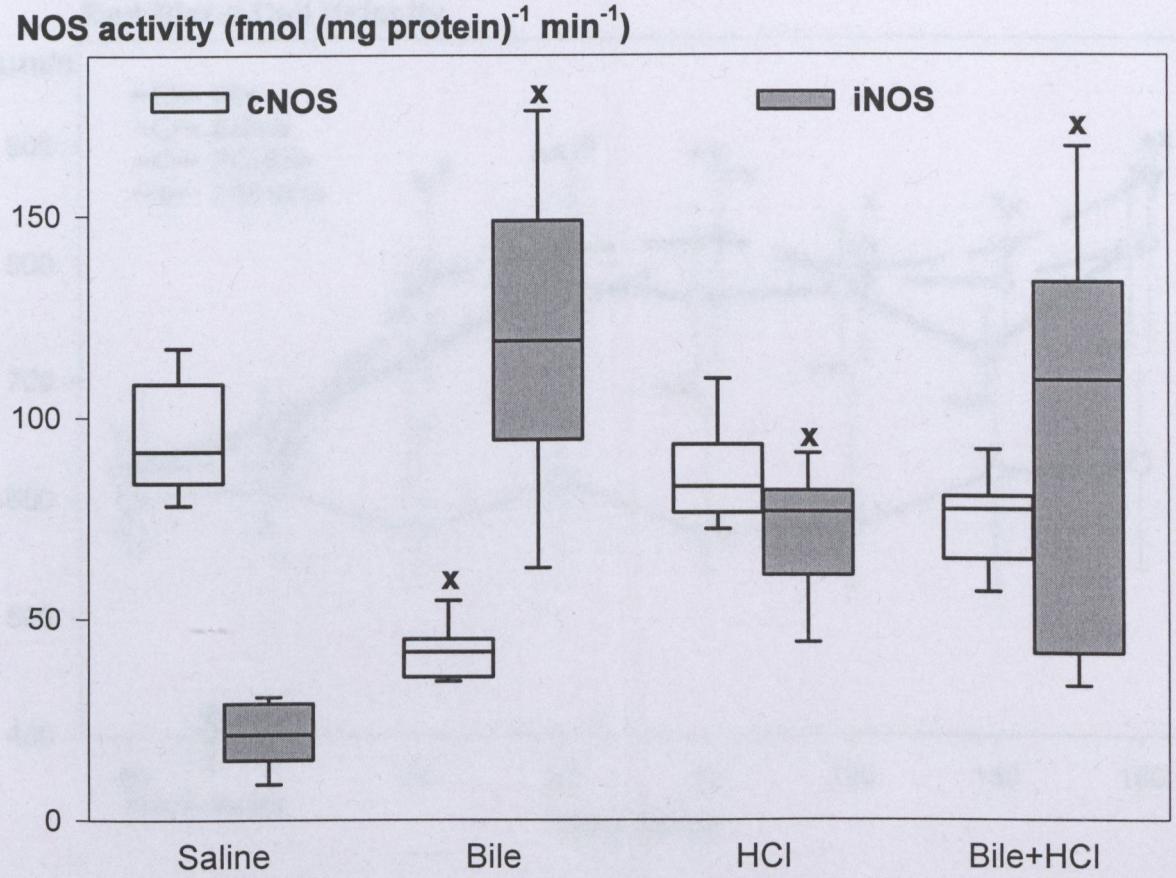

Figure 8. Changes in constitutive (empty column) and inducible (shaded column) NOS activities (fmol (mg protein^{-1} min^{-1}) in esophageal tissue from saline-treated (n=5), bile-treated (n=8), HCl-treated (n=5) and bile + HCl treated (n=5) animals. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles, respectively. X: p<0.05 between groups *vs* saline-treated control group values.

4.2. STUDY II

The baseline values of the macrohemodynamic variables did not differ significantly in the different groups and there were no significant hemodynamic changes as compared with the control values during the experimental period. The MAP in the bile+7-NI or PC-treated groups was not significantly different (p>0.05) from that for the saline-treated group as a whole.

The baseline RBCV level in the various groups ranged between 560 and 680 μm s^{-1}, and in the control group the RBCV did not change during the experiments. However, the RBCV was increased significantly after the 3-h exposure to bile with or without 7-NI or PC pretreatment. The median values were 813, 886 and 787 μm s^{-1} after bile, after bile with 7-NI, and after bile with PC pretreatment, respectively (Figure 9).

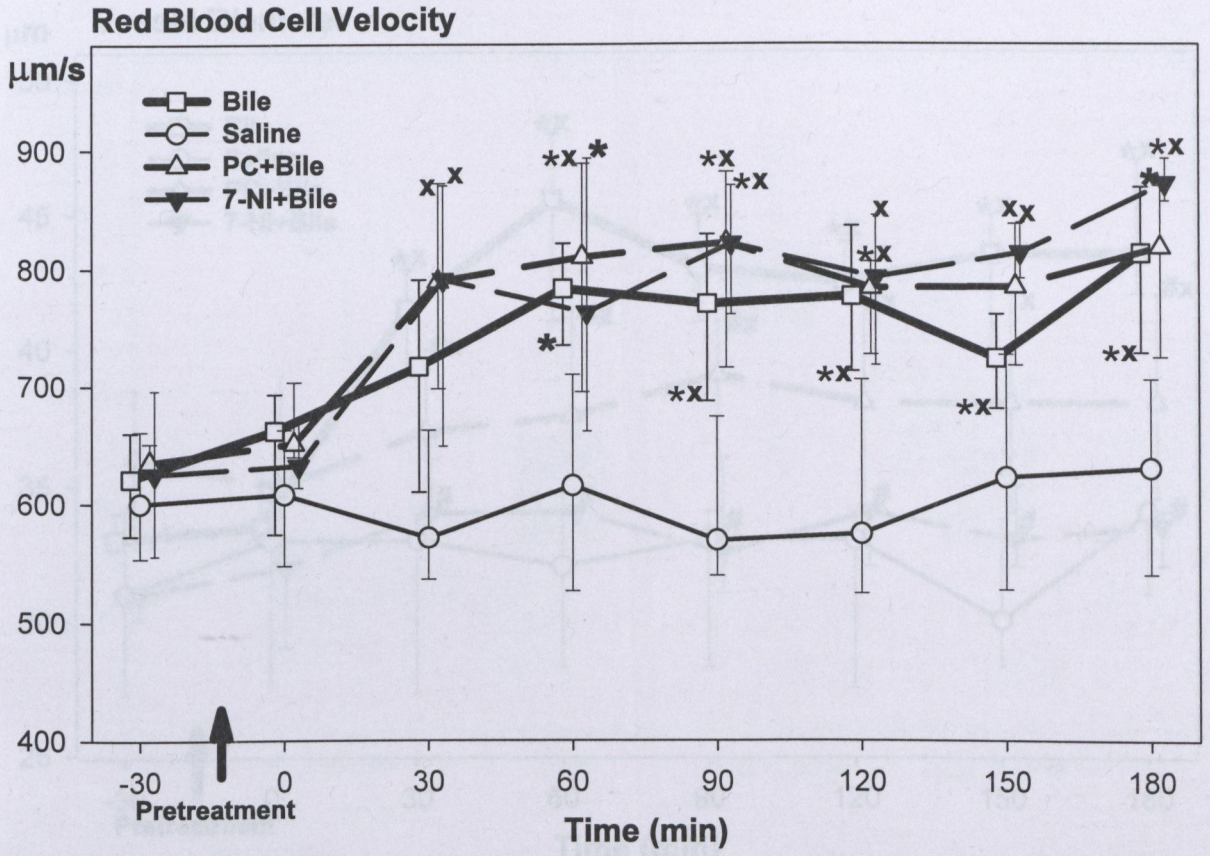

Figure 9. Effects of different intraluminar treatments and systemic pretreatments on the red blood cell velocity (RBCV; μm s^{-1}). Saline treatment (thin line with empty circles), bile treatment alone (thick line with empty squares), bile treatment with PC pretreatment (thick line with empty triangles) and bile treatment with 7-NI pretreatment (thin line with full triangles) groups. *: $p<0.05$ within groups *vs* baseline values; X: $p<0.05$ between groups *vs* saline-treated control group values.

Venules 35±10 μm in diameter were the largest fraction of the vessels; arterioles were seen only very rarely. The inner boundary of the venular wall was easily distinguishable and an exact comparison of inner diameter changes was possible. The median VD was significantly higher in the bile-treated group as compared with the saline-treated control, and increased from the baseline value of 33 μm to 44 μm. Administration of 7-NI before bile treatment prevented this increase. PC pretreatment resulted in a significant VD elevation as compared with the control group, but the VD changes in this group were significantly lower than those in the bile-treated group (Figure 10).

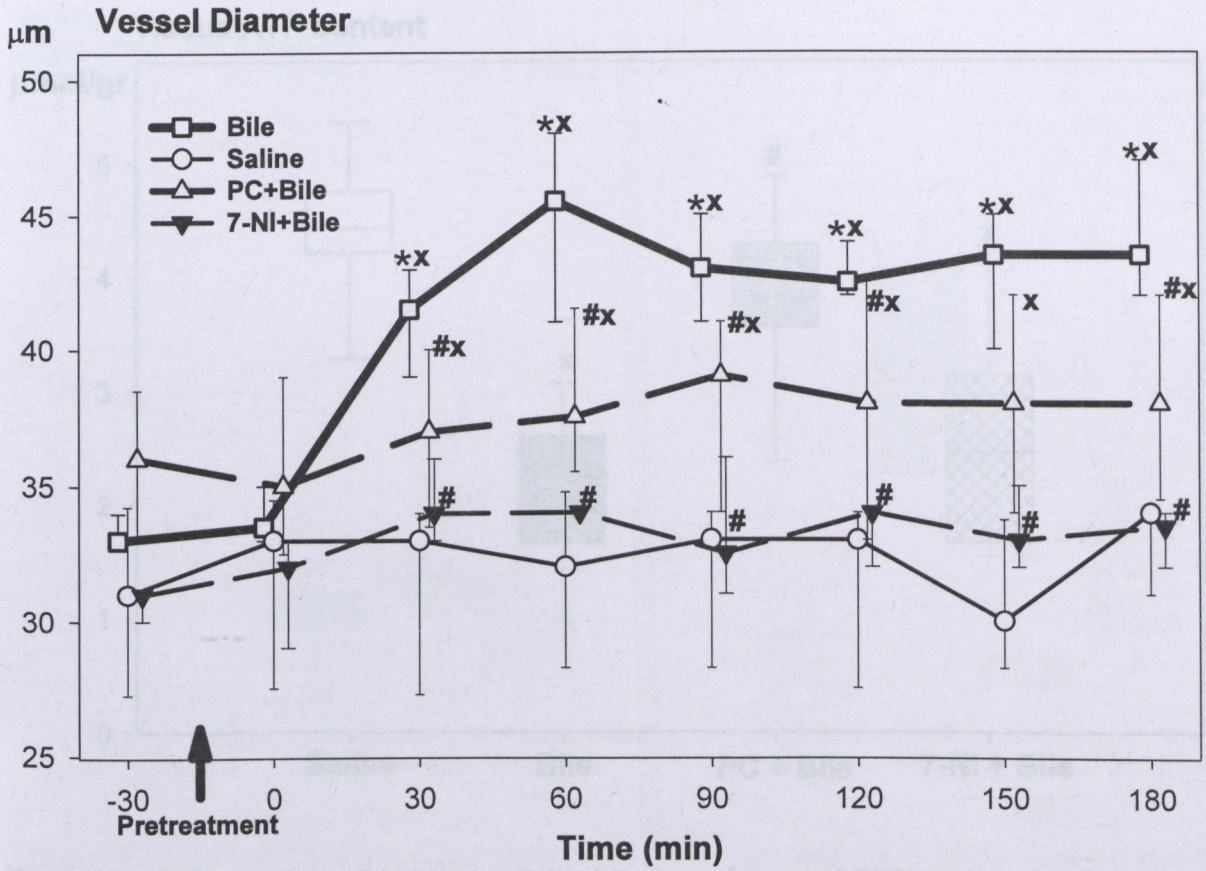

Figure 10. Changes in vessel diameter (VD; μm). Saline treated (thin line with empty circles), bile-treated (thick line with empty squares), bile + PC-treated (thick line with empty triangles) and bile + 7-NI-treated (thin line with full triangles) groups. *: $p<0.05$ within groups *vs* baseline values; X: $p<0.05$ between groups *vs* saline-treated control group values.

The *in vivo* interference of the bile treatment with the ATP production of the esophageal mucosa was evaluated (Figure 11). There were no significant differences in tissue ATP levels between the intact and treated parts of the esophagus in the saline-treated control group (data not shown). There was a statistically significant, 45% fall in the ATP content of the esophageal tissue after bile treatment (saline: M=4.43 μmol mg protein^{-1}; 25p=4.21; 75p=4.76; bile: M=2.42 μmol mg protein^{-1}; 25p=1.66; 75p=2.61) by the end of the observation period, and this change was not affected by 7-NI treatment. Following PC treatment, the ATP content was significantly maintained in the mucosa as compared with bile treatment alone (M=3.82; 25p=3.56; 75p=4.28), and this value was not significantly different from the corresponding value for the saline-treated group.

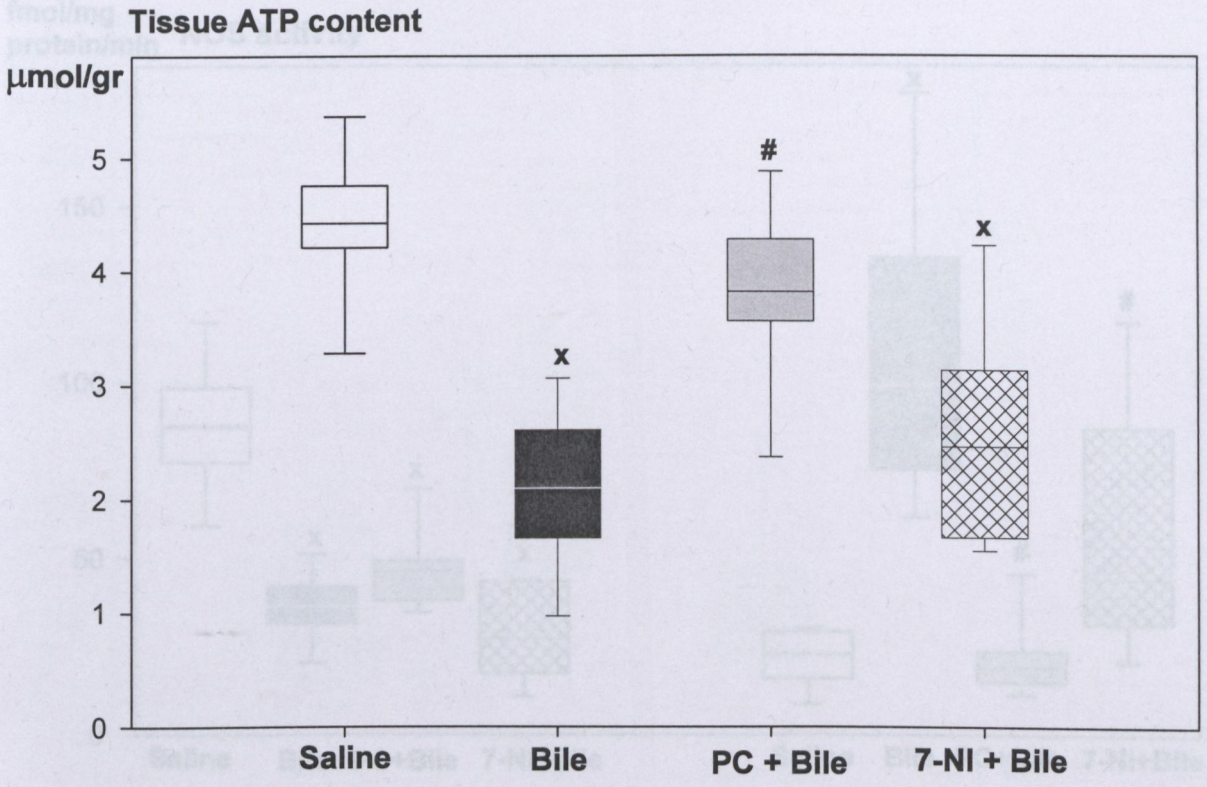

Figure 11. Effects of bile (black box), PC + bile (gray box) and 7-NI + bile (checked box) on the ATP content of the esophageal mucosa. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles, respectively. X: $p<0.05$ between groups *vs* saline-treated control group values, #: $p<0.05$ between bile + 7-NI-treated and bile + PC-treated groups *vs* bile-treated group values.

Figure 12 demonstrates the changes in the esophageal cNOS and iNOS activities. The activity of cNOS was significantly depressed after bile treatment, and this change was accompanied by a significant, 6-fold increase in iNOS activity. The cNOS activity was not altered by PC or 7-NI pretreatment. However, the esophageal iNOS activity was significantly lower after PC pretreatment as compared with bile treatment alone. 7-NI treatment resulted in a somewhat lower, 2.6-fold increase in iNOS activity as compared with the value for the bile-treated group.

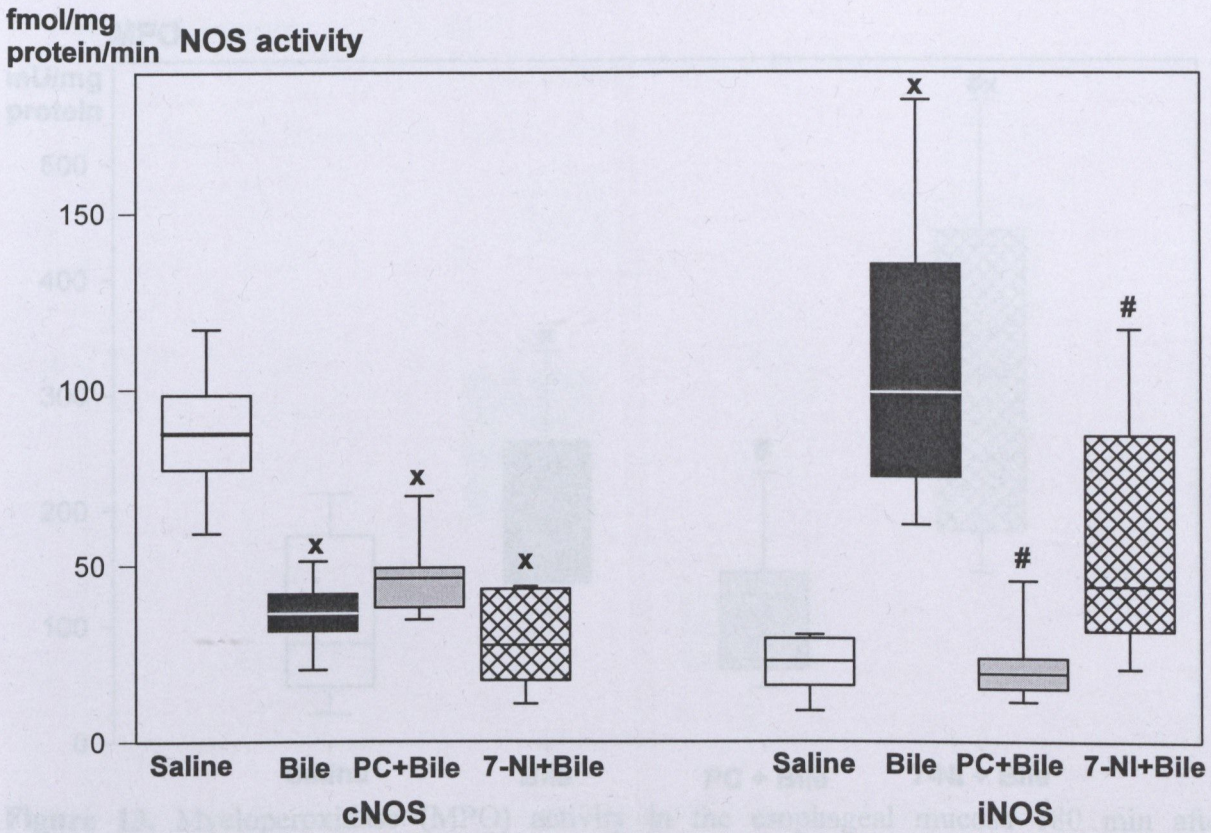

Figure 12. Changes in constitutive (left panel) and inducible NOS (right panel) activities (fmol (mg protein)$^{-1}$ min^{-1}) in esophageal tissue from saline-treated (empty box), bile-treated (black box), bile + PC-treated (gray box) and bile + 7-NI-treated (checked box) animals. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles, respectively. X: $p<0.05$ between groups *vs* saline-treated control group values, #: $p<0.05$ between bile + 7-NI-treated and bile + PC-treated groups *vs* bile-treated group values.

The MPO data demonstrated that the leukocyte accumulation was significantly increased in the mucosa in the bile-treated and 7-NI-pretreated groups as compared with the saline-treated group (Figure 13). Bile alone resulted in a 2.5-fold (M=209.4; 25p=136.6; 75p=259.6) rise in MPO activity, and a further increase was observed after 7-NI + bile administration (M=315.4; 25p=182.4; 75p=441.8). PC pretreatment significantly decreased the bile-induced MPO activity.

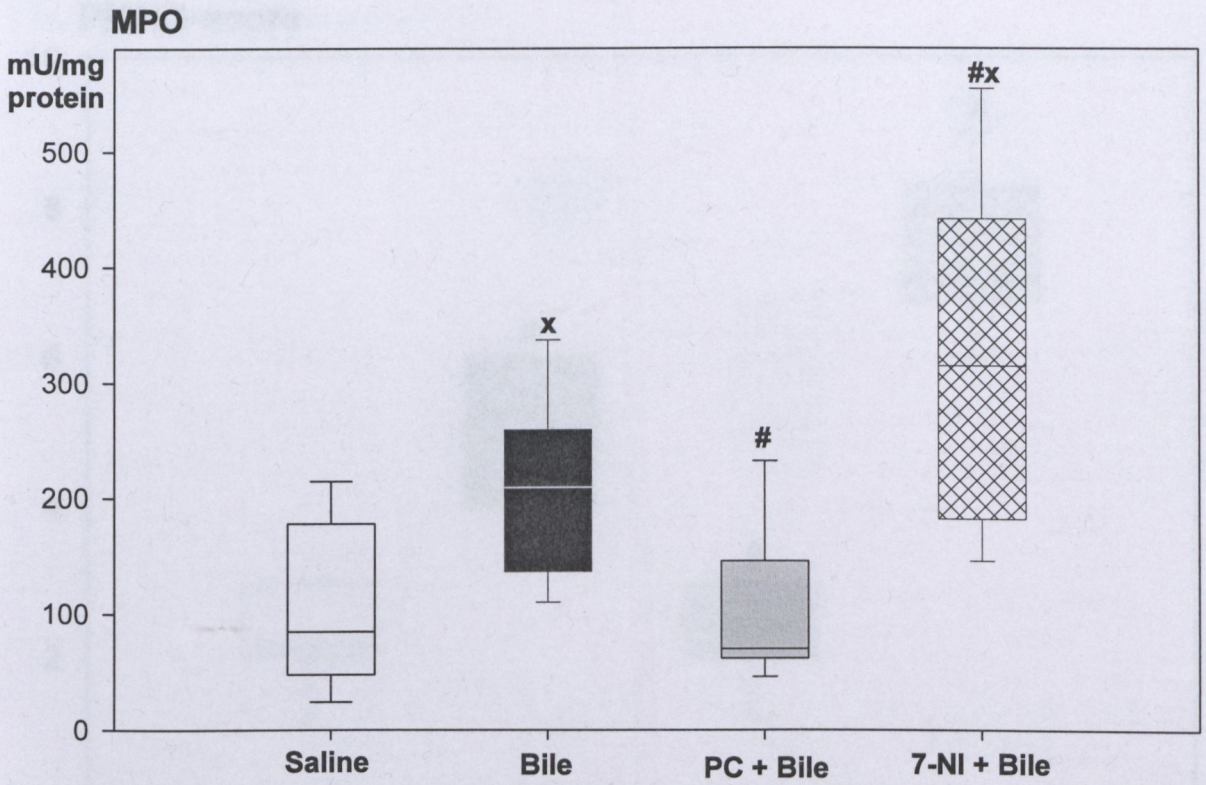

Figure 13. Myeloperoxidase (MPO) activity in the esophageal mucosa 180 min after treatment. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles, respectively. X: $p<0.05$ between groups *vs* saline-treated control group values, #: $p<0.05$ between bile + 7-NI-treated and bile + PC-treated groups *vs* bile-treated group values.

Histological scoring of leukocyte infiltration was performed by two independent viewers (the inter-observer variation was less than 15%, and these data showed a good correlation with the MPO results). In the saline-treated group, the extravasation of PMNs was negligible (range of scores: 1-3; M=2). Exposure to bile resulted in a significant ($p<0.05$) accumulation of PMNs (range: 4-6; M=5). In the PC-pretreated animals, the PMN infiltration was significantly decreased and the scores not differ significantly from the control values (range: 1-3; M=3). Bile + 7-NI treatment increased the degree of PMN infiltration and resulted in a grade 7 score (range: 7-9) (Figure 14).

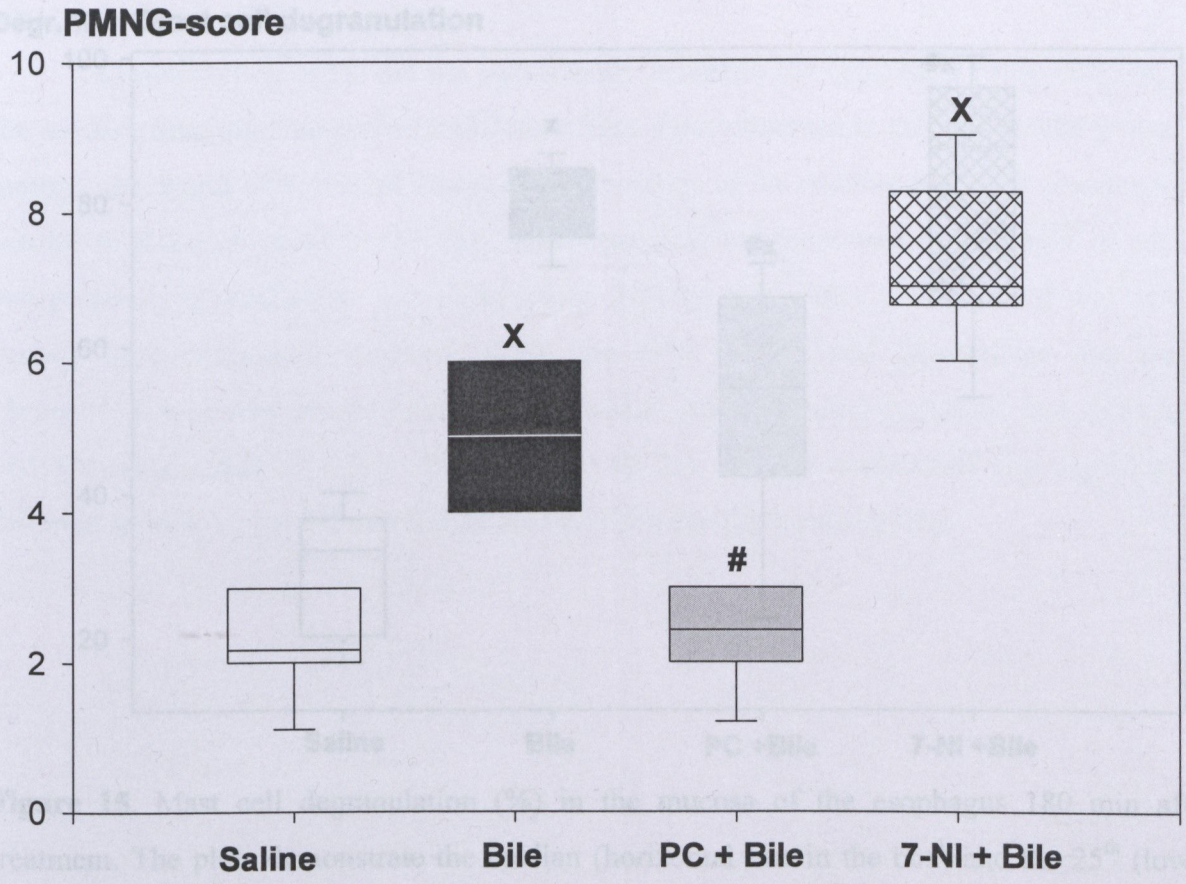

Figure 14. Scores of leukocyte infiltration of esophageal tissue after different intraluminar treatments and systemic pretreatments with grading according to our scoring system (for a description, see the Methods). The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles, respectively. X: $p<0.05$ between groups *vs* saline-treated control group values, #: $p<0.05$ between bile + 7-NI-treated and bile + PC-treated groups *vs*,bile-treated group values.

The effects of bile and various pretreatments on the mucosal MC degranulation are presented in Figure 15. In the control group, 35% of the MCs had degranulated. Exposure to bile resulted in a significant, degranulation (M=79.6%; 25p=75.0; 75p=84.6) relative to the control group. In the PC-pretreated group, the percentage of degranulated MCs (M=54.3%, 25p=42.2%; 75p=66.7%) proved to be significantly lower than that after bile treatment alone. The nNOS inhibition evoked a marked stimulation of MC degranulation over that due to bile treatment alone. In the 7-NI + bile-treated group, the degranulation of the mucosal MCs was nearly complete (M=87.5%; 25p=65.6%; 75p=95.6%).

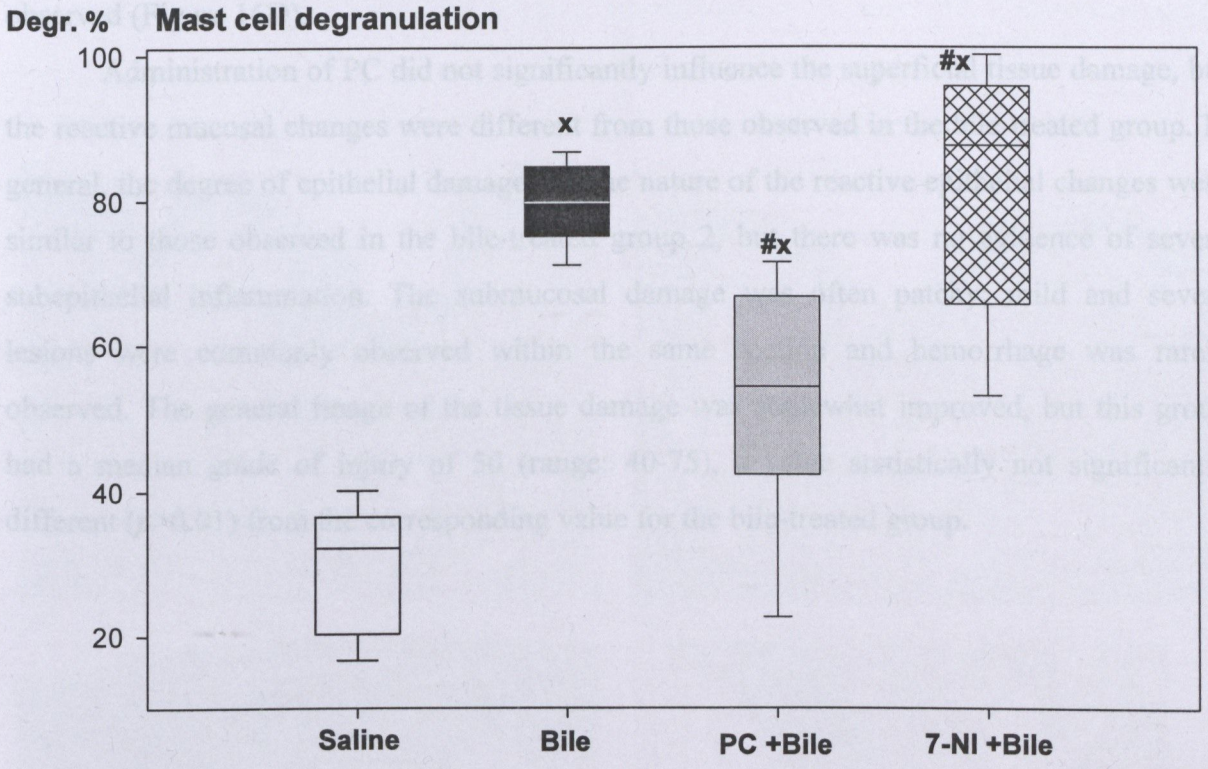

Figure 15. Mast cell degranulation (%) in the mucosa of the esophagus 180 min after treatment. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles. $p<0.05$ between groups *vs* saline-treated control group values. $p<0.05$ between bile + 7-NI-treated and bile + PC-treated groups *vs* bile-treated group values.

The biopsy samples from the saline-treated control group exhibited an average grade of injury of 5 (range of scores: 0-20) on the Lanas scale. In these sections, the luminal surface was always lined by a continuous layer of epithelial cells, while the vessels usually presented with empty lumina (Figure 16 A). The 180-min bile exposure induced significant mucosal damage ($p<0.01$), with a median value of 58 (range: 50-70). Deep lesions were observed, with disruption and desquamation of the epithelial layer (Figure 16B,C). Extensive intraepithelial and subepithelial leukocytosis and connective tissue damage were general characteristics. Submucosal edema, hemorrhage and vasodilatation were apparent. Semiquantitative evaluation of the samples from the 7-NI + bile-treated animals revealed a significant exacerbation of the mucosal injury ($p<0.01$) and an injury score of 68 (range 60-90). A tendency toward more intense injury was always manifested. Severe epithelial damage and exfoliation of the surface epithelium were present, and the deeper tissue layers were more strongly involved in a generalized inflammatory reaction. In most cases, transmural inflammatory infiltrations, vasodilation and subepithelial connective tissue edema were

observed (Figure 16D).

Administration of PC did not significantly influence the superficial tissue damage, but the reactive mucosal changes were different from those observed in the bile-treated group. In general, the degree of epithelial damage and the nature of the reactive epithelial changes were similar to those observed in the bile-treated group 2, but there was no evidence of severe subepithelial inflammation. The submucosal damage was often patchy; mild and severe lesions were commonly observed within the same section and hemorrhage was rarely observed. The general image of the tissue damage was somewhat improved, but this group had a median grade of injury of 50 (range: 40-75), a value statistically not significantly different ($p>0.01$) from the corresponding value for the bile-treated group.

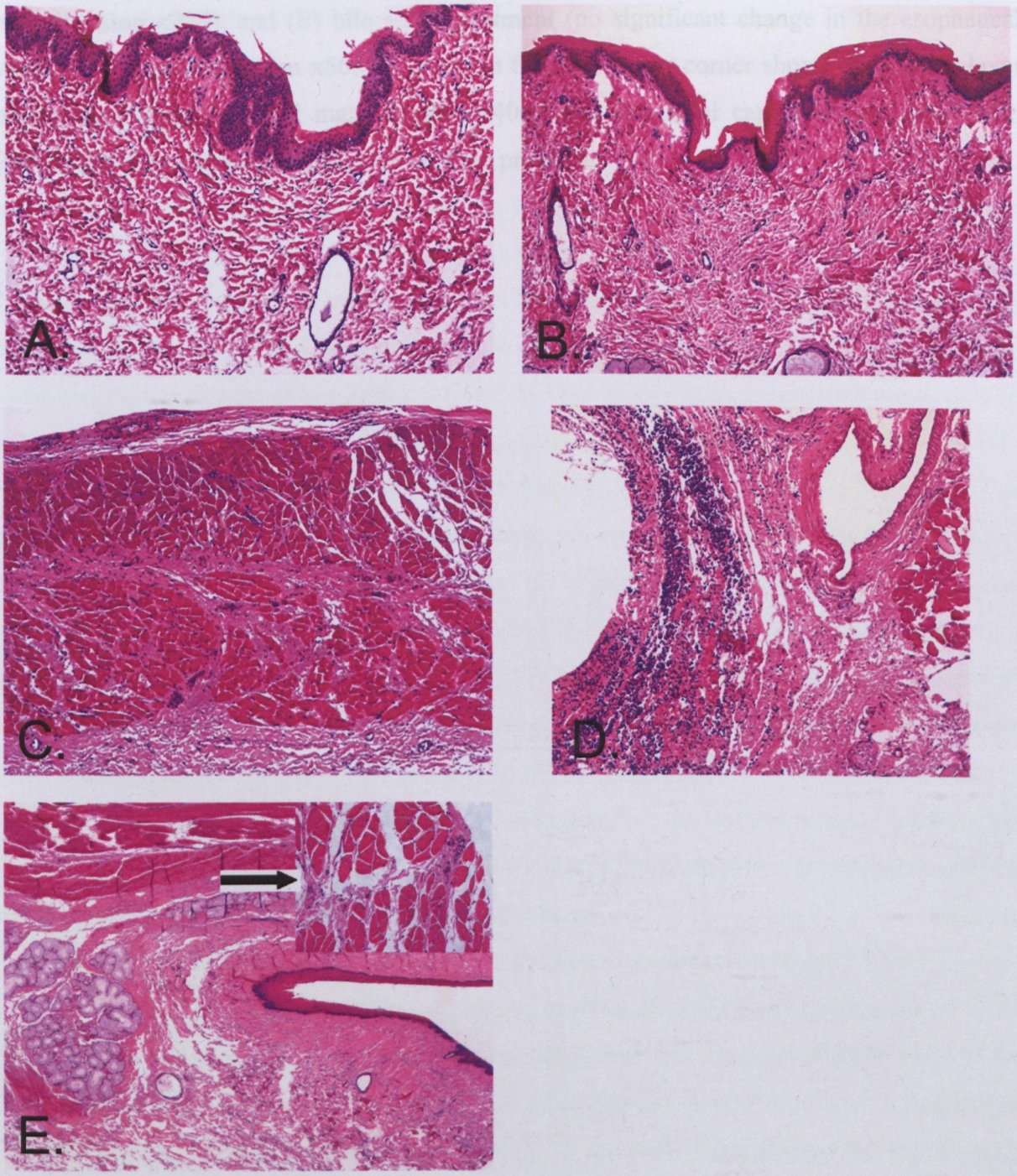

Figure 16. Photomicrographs of the esophageal wall from groups 1-4 (H&E staining). (A) control group (saline treatment, original magnification x 224); (B) bile treatment (epithelial

damage and subepithelial edema, original magnification x 112); (C) bile-treated group (significant leukocyte infiltration in the muscle layers and adventitia, original magnification x112); (D) bile+7-NI treatment (extreme leukocyte infiltration in the adventitia, original magnification x224); and (E) bile + PC treatment (no significant change in the esophageal wall, original magnification x56; the arrow in the right upper corner shows the muscle layer of the same field (original magnification x400); the interstitial capillaries are filled with PMNs, but only a few extravasated PMNs are present).

5. DISCUSSION

GERD is the most frequent inflammatory disorder of the foregut. During recent decades, the importance of this disease has increased steadily, and it now influences a high percentage of the population, with negative effects on the health-related quality of life. GERD is also a risk factor for Barret's esophagus and esophageal adenocarcinoma formation. Chronic exposure to the duodenogastric content frequently leads to an esophageal dysfunction and tissue damage, but an acute reflux may likewise result in severe esophagitis and esophageal ulceration in critically ill, mechanically ventilated patients.

A number of studies have emphasized the impact of duodenal components in the progression of GERD, and duodenogastroesophageal or alkaline reflux has become an autonomic entity (**Vaezi *et al.*, 2001**). At first sight, a biliary reflux alone can not evolve in GERD patients, because it is obviously associated with acidic regurgitation. However, HCl secretion can be completely blocked by PPI therapy. Gastric acid-suppressive therapy or achlorhydria may lead to bacterial colonization in the upper gastrointestinal tract (**Drasar *et al.*, 1969**). As a result of the bacterial flora, deconjugated bile acids appear in a higher proportion, including severe mucosal damage in the esophagus (**Salo *et al.*, 1983**). Thus, in patients on PPI therapy with bile regurgitation, a "bile danger zone" of higher pH (pH>5.0) has to be considered. These findings underline that pH-metry alone, which was earlier thought to be a reliable diagnostic method, is not able to prove the presence of GERD in every case.

The significance of biliary reflux and the need for appropriate medical treatment is indisputable. Accordingly, the first step should be a comprehensive investigation of the pathomechanism to trace the possible targets of therapy.

In the first part of our *in vivo* study, the separate responses to HCl and bile were compared. The early microcirculatory consequences of acidic and biliary exposure were quite similar. It was recognized that microcirculatory changes can be considered to be parts of the local inflammation, and the symptoms are not influenced by macrohemodynamic variables. These microcirculatory changes appeared quickly as the RBCV and RVA were significantly increased 90 min after the start of the treatment. The RBCV is determined primarily by the blood flow and the cross-section of the circulatory area, and this suggests that significant vasodilation evolves in the mucosa. The elevated RVA values provided direct evidence of the presence of vasodilation.

The results of biochemical measurements may explain the microvascular changes. Exposure to either HCl or bile was accompanied by a significant rise in tissue MPO activity.

This phenomen is a good marker of leukocyte accumulation. The contact of the neutrophilic leukocyte with a chemoattractant triggers a complex metabolic event, with the production of oxygen intermediates. Moreover, a variety of inflammatory mediators could be released from these cells. Once the leukocytes have entered the tissues, they may induce further mucosal and submucosal damage, thereby mediating the aggressive action of intraluminal agents. These data suggest that leukocyte activation and tissue accumulation are important components of mucosal destruction in both biliary and acidic reflux.

Comparison of the structural changes caused by the observed agents revealed differences between bile- and acid-induced inflammation. Severe tissue damage was characteristic for both groups, but exposure to bile resulted in more serious mucosal and submucosal injuries. In this regard, the histological results required a further explanation in order to allow an understanding of the difference in the pathomechanisms and to acquire valid conclusions from studies involving tissue salvage therapies.

The toxicity of bile acids has already been demonstrated in hepatic tissue. It has also been confirmed that the intrahepatic accumulation of bile salts is directly connected with a mitochondrial dysfunction (**Schmucker *et al.*, 1990, Krahenbuhl *et al.*, 1994**). Bile salts may cause hepatocyte death by inducing mitochondrial permeability transition, Fas-dependent hepatocyte apoptosis or necrosis. Bile salts at low concentrations inhibit the activities of complexes I and III of the mitochondrial respiratory chain (**Rosser *et al.*, 1995**). It has been proved that bile, with or without gastric acid, leads to ATP depletion in esophageal tissue. A recent study concluded that ATP depletion of the canine esophagus after biliary exposure may be a result of a dramatic transcriptional level downregulation of mucosal and smooth muscle βATPase gene expression (**Szentpáli *et al.*, 2005**). However, this change was not observed when the esophagus was exposed to HCl alone (**Szentpáli *et al.*, 2001**). In line with these data, a possible reason for the described morphological differences was found. These findings emphasize the importance of monitoring of other functional parameters.

In the relevant literature a number of data underline the role of NO. This molecule is formed *in vivo* by the continuously active cNOS, and is involved in the physiological regulation of the peripheral vascular tone (**Moncada *et al.*, 1991**). In inflammation, endotoxemia or sepsis, a distinct form, the inducible, Ca^{2+}-independent iNOS, is activated, thereby leading to an excessive production of NO (**Zhang *et al.* 1998**). NO plays a central role in the maintenance of the normal resting esophageal mucosal blood flow, and an increased esophageal blood flow may have a protective role against damaging gastric juice or other noxious stimuli (**Sandler *et al.*, 1993, McKie *et al.*, 1995**). In our study, the bile-

induced microcirculatory alterations were accompanied by inverse changes in the cNOS and iNOS activities. Although gastric acid alone also increased the activity of iNOS, it did not influence the cNOS activity. This finding suggests that the activation of iNOS is a consequence of an inflammatory reaction in cases of acidic reflux. The effect of bile on NO production seems to be much more complex. The connection between bile and the cNOS activity has been described in biliary fibrosis and after experimental bile-duct ligation (**Zimmermann *et al.*, 1996, Rockey *et al.*, 1998, Wei *et al.*, 2002**). It has also been reported that a low cNOS and an increased iNOS activity are responsible for the exacerbation of gastric injury from luminal irritants during endotoxemia (**Helmer *et al.*, 2002**). Accordingly, we can presume that the higher iNOS activity is a compensatory event of cNOS inhibition, or an aftermath of inflammatory stimuli. Moreover, it has been described that the expression of iNOS is increased in taurocholate-induced pancreatitis **(Satoh *et al.*, 1998)**. Although the lowered ATP generation could be an important factor in the acute decrease in cNOS activity, the intracellular biochemical mechanisms that mediate this injury are not completely understood. It has been speculated that unconjugated di- and trihydroxy bile acids cause damage by binding to and across cell membranes to enter cells. However, it has recently been shown that the β-chain of ATP synthase, a principal protein complex in the mitochondrial inner membrane, is also present at the cellular surface and plays a decisive role in the regulation of cell homeostasis. As already mentioned, bile acids inhibit the activities of complexes I and III of the mitochondrial respiratory chain and may cause cytochrome c release and Fas-dependent apoptosis or necrosis. Additionally, bile acids have a direct toxic effect on the mitochondria (**Krahenbuhl *et al.*, 1994**).

In our model, exposure to different noxious agents was examined over a relatively short time, but long-term effects of NO have to be considered too. GERD is a chronic inflammatory disease, which is linked to carcinogenesis. The frequency of adenocarcinoma is increased in patients with biliary reflux and chronic inflammation itself can lead to malignant transformation (**Fein *et al.*, 2000**). Other investigators have suggested that the mechanism of the mitogenic effects of bile acids may involve the N-nitrosation of glycine and taurine amides, leading to the production of carcinogenic N-nitroso amides (**Dayal *et al.*, 1997**). Beside its protective effects, excess NO generation is likely to contribute directly to the formation of N-nitroso compounds. Thus, the bile-induced overproduction of NO in the long run presumably plays a role in the mutagenic process (**Bartsch *et al.*, 1992**).

In the second part of our study, we obtained more data on the pathomechanism of biliary esophagitis. The cNOS activity, which was measured in the course of our

examinations, involves the activities of two different isoforms: eNOS and nNOS. It has been shown that the nNOS activity is responsible for more than 90% of this activity in the gastrointestinal tract. Further, the expression of nNOS protein is the highest in the esophagus (**Fischer *et al.*, 1999, Qu *et al.*, 1999**). Additional connections between the iNOS and nNOS activities were recently described as the function of inducible, Ca^{2+}-independent iNOS is closely related to the activation of NF-κB. In the small intestine nNOS suppresses the gene expression of iNOS through NF-κB downregulation, while nNOS suppression leads to NF-κB activation and iNOS expression (**Qu *et al.*, 2001**). These data provided an explanation for our results and emphasize the importance of nNOS-derived NO in bile-induced esophageal inflammation.

We presumed that neutrophilic leukocytes are not the only cellular effectors of this inflammatory process. The results showed that MCs too are important factors in the pathomechanism of inflammation. Mucosal MCs are a unique source of both preformed and *de novo* synthesized mediators, and MC-induced reactions contribute to postischemic mucosal permeability alterations and flow responses in the gastrointestinal tract (**Szabo *et al.*, 1997, Boros *et al.*, 2003**). Bile acids are able to degranulate MCs both *in vitro* and *in vivo* (**Rees *et al.*, 1978, Quist *et al.*, 1991**). Moreover, they induce secretion in the small intestine by a mechanism involving a histamine-mediated process and MC degranulation (**Gelbmann *et al.*, 1995, Hardcastle *et al.*, 2001**). It has further been reported that MC-derived histamine is involved in esophageal and gastric vasodilation during acid-induced injury (**Feldman *et al.*, 1996**). This type of vasodilation could protect the mucosa against further injury and appears to be mediated by calcitonin gene-related peptides (**McKie *et al.*, 1994**). Thus, it is reasonable to suggest that the bile-induced venodilatory response is closely associated with MC degranulation.

In our study, 7-NI pretreatment significantly amplified the bile-induced tissue damage and caused further MC degranulation. 7-NI is an *in vivo* selective inhibitor of nNOS, because it is selectively taken up by cells expressing the nNOS isoform. 7-NI inhibits eNOS only at high concentrations (**Southan *et al.*, 1996, Ayajiki *et al.*, 2001**). The residual responses to 7-NI might be mediated by a previously unblocked part of the nNOS isoform. This also suggests that bile principally inhibits eNOS, and the partially sustained nNOS activity is able to counteract or at least diminish the harmful effects of bile. It was revealed several years ago that NO has an inhibitory action on histamine release from MCs (**Masini *et al.*, 1991, Salvemini *et al.*, 1991,**). Thus, the decreased activity of cNOS isoforms may result in the degranulation of a high percentage of the esophageal MCs. However, eNOS and nNOS

inhibition alone can not explain our results, because the activation of iNOS could probably compensate for the absence of cNOS-derived NO. It has been described that MCs in the gastrointestinal tract interact directly with nerve cells (**Bauer *et al.*, 2000**). As regards the high level of expression of nNOS in the esophagus, we can presume the direct role of this isoform in the attenuation of MC activation. This underlines the role of nNOS-derived NO in the maintenance of esophageal homeostasis.

In the PC-pretreated animals, the bile-elicited ATP decrease, cNOS inhibiton and MC degranulation were significantly attenuated. The question therefore arises as to which process may be of critical significance in the mechanism of mucosal protection after PC treatment. PC is a ubiquitous membrane-forming entity and the most prominent phospholipid species in the gastrointestinal tract. PC is present in high concentration in the bile and constitutes 40% of the organic material of bile. *In vitro* and *in vivo* experiments have demonstrated that topical PC protects the intestinal mucosa physically against the injurious actions of bile salts by forming less toxic mixed micelles (**Barrios *et al.*, 2000**). Nevertheless, the experimental results and clinical experience suggest that PC could function as an active substance under certain *in vivo* conditions. The therapeutic effect of dietary PC in preventing esophageal strictures due to alkali-induced esophageal burns has been demonstrated in rats (**Demirbilek *et al.*, 2002**), and parenteral PC and lyso-PC prolonged survival in experimental sepsis models (**Drobnik *et al.*, 2003, Yan *et al.*, 2004**). PC is taken up by phagocytic cells and accumulates in inflamed tissues (**Cleland *et al.*, 1979**). Little is known about the transport of the molecule, but the PC transfer protein accelerates the intermembrane transfer of phospholipids in an energy-independent way. Choline, a metabolite of PC and a precursor of organic osmolyte betaine, is actively transported by a choline carrier described in intestinal epithelial and endothelial cells of the blood-brain barrier (**Friedrich *et al.*, 2001**).

It is widely believed that the biological efficacy of PC depends on the fatty acid moiety (**Lieber *et al.*, 1997**). In contrast, some studies have revealed that the protective role of PC is independent of fatty acids, and it may be assumed that the active principle is choline. Phospholipase-D is activated by almost all stress factors resulting in the release of phospholipid metabolites, and several of these factors could be of importance in stress-induced defense reactions (**Exton, 1999**). Indeed, it has been shown that PC metabolites might relieve a potentially dangerous increase in the ratio of NADH / NAD^+ (reductive stress), a predisposing cause of oxidative damage (**Ghyczy *et al.*, 2003**). This reaction sequence could explain the still incompletely understood essential role of choline in diet, and its preventive efficacy in a number of experimentally induced pathologies associated with a

redox imbalance. It may be assumed that the endogenous pool of these metabolites may become exhausted during exogenous provocation and that an exogenous supply might help to replenish and strengthen the endogenous protective mechanism.

In conclusion, our data suggest that the nNOS-MC axis plays an important role in the mucosal defense system of the esophagus. Elucidation of the mechanisms by which bile acids induce a mucosal dysfunction is complicated by the intrinsic complexity of the esophageal tissue, which is made up of many different, but interacting cell types. Whether the findings in this experimental model are applicable to humans remains to be established. However, these data together with previous observations suggest a therapeutic potential for parenteral PC with a view to decreasing the harmful consequences on bile-induced tissue reactions.

6. SUMMARY AND NEW FINDINGS

Our *in vivo* experimental model has provided information on the functional, morphological and biochemical changes during the initiation phase of acute esophagitis. The investigations have shown that exposure to bile or gastric acid results in local vasodilation, an increased capillary flow and leukocyte accumulation.

- The histological and biochemical findings proved that bile causes a more severe mucosal impairment than that due to HCl alone.
- Our study has proved that NO and different isoforms of NOS play important roles in the pathomechanism of esophagitis.
- nNOS, as the predominant cNOS isoform in the esophagus, is able to protect the esophageal mucosa by preventing the degranulation of mast cells.
- MC degranulation is involved in the multiplex, noxious effects of bile.
- Selective inhibition of cNOS exacerbates the tissue damage.

We presumed that PC might ameliorate bile-induced inflammation.

- Our results indicate that PC treatment prevents leukocyte accumulation, decreases excessive NO production and MC degranulation, and mitigates the bile-induced morphological changes.

These findings reveal the importance of the nNOS-MC axis in the mucosal defense system of the esophagus, and additionally that PC may comprise new, prospective therapy for biliary esophagitis.

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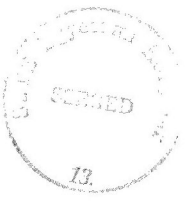

8. REFERENCES

1) Attwood SEA, DeMeester TR, Bremner CG, Barlow AP, Hinder RA. Alkaline gastroesophageal reflux: implication in the development of complications of Barrett's columnar lined esophagus. Surgery 1989; 106: 764-70.
2) Ayajiki K, Fujioka H, Okamura T, Toda N. Relatively selective neuronal nitric oxide synthase inhibition by nitroindazole in monkey isolated cerebral arteries. Eur J Pharmacol 2001; 423(2-3): 179-83.
3) Barrios JM, Lichtenberger LM. Role of biliary phosphatidylcholine in bile acid protection and NSAID injury of the ileal mucosa in rats. Gastroenterology 2000; 118: 1179-1186.
4) Bartsch H, Ohshima H, Pignatelli B, Calmels S. Endogenously formed N-nitroso compounds and nitrosating agents in human cancer etiology. Pharmacogenetics 1992; 2: 272-7.
5) Bauer O, Razin E. Mast cell-nerve interactions. NIPS 2000; 15: 213-8.
6) Boros M. Microcirculatory dysfunction during intestinal ischemia-reperfusion. Acta Physiol Hung 2003; 90: 263-279.
7) Bremner CG, Mason RJ. Bile in esophagus. Br J Surg 1993; 80: 1374-6.
8) Buchman AL, Dubin MD, Moukarzel AA et al. Choline deficiency; a cause of hepatic steatosis during parenteral nutrition that can be reversed with intravenous choline supplementation. Hepatology 1995; 22: 1399-1403.
9) Cleland LG, Shandling M, Percy JS, Poznansky MJ. Liposomes: a new approach to gold therapy? J Rheumatol Suppl 1979; 5: 154-163.
10) Dayal B, Ertel NH. Studies on N-nitroso bile acid amides in relation to their possible role in gastrointestinal cancer. Lipids 1997; 32: 1331-40.
11) DeMeester SR, DeMeester TR. Columnar mucosa and intestinal metaplasia of the esophagus: fifty years of controversy. Ann Surg 2000; 231: 303-321.
12) Devesa SS, Blot WJ, Fraumeni JF Jr. Changing patterns in the incidence of esophageal and gastric carcinoma in the United States. Cancer 1998; 83: 2049-2053.
13) Demirbilek S, Aydin G, Yucesan S, Vural H, Bitiren M. Polyunsaturated phosphatidylcholine lowers collagen deposition in a rat model of corrosive esophageal burn. Eur J Pediatr Surg 2002; 12: 8-12.

14) Drasar BS, Shiner M, McLeord GM. Studies on the intestinal flora. I. The bacterial flora of the gastrointestinal tract in healthy and achlorhydric persons. Gastroenterology 1969; 56: 71-79.

15) Drobnik W, Liebisch G, Audebert FX, Frohlich D, Gluck T, Vogel P, Rothe G, Schmitz G. Plasma ceramide and lysophophatidylcholine inversely correlate with mortality in sepsis patients. J Lipid Res 2003; 44: 754-761.

16) el-Hariri LM, Marriott C, Martin GP. The mitigating effects of phosphatidylcholines on bile salt- and lysophosphatidylcholine-induced membrane damage. J Pharm Pharmacol 1992; 44: 651-654.

17) Exton JH. Regulation of phospholipase D. Biochim Biophys Acta 1999; 1439: 121-133.

18) Fein M, Fuchs KH, Stopper H, Diem S, Herderich M. Duodenogastric reflux and foregut carcinogenesis: analysis of duodenal juice in a rodent model of cancer. Carcinogenesis 2000; 21: 2079-84.

19) Feldman MJ, Morris GP, Dinda PK, Paterson WG. Mast cell mediate acid-induced augmentation of opossum esophageal blood flow via histamine and nitric oxide. Gastroenterology 1996; 110: 121-8.

20) Fischer H, Becker JC, Boknik P, Huber V, Luss H, Neumann J, Schmitz W, Domschke W, Stachura J, Konturek JW. Expression of constitutive nitric oxide synthase in rat and human gastrointestinal tract. Biochem Biophys Acta 1999; 1450: 414-422.

21) Friedrich A, George RL, Bridges CC, Prasad PD, Ganapathy V. Transport of choline and its relationship to the expression of the organic cation transporters in a rat brain microvessel endothelial cell line (RBE4). Biochim Biophys Acta 2001; 1512: 299-307.

22) Fujiwara Y, Higuchi K, Watanabe Y, Shiba M, Watanabe T, Tominaga K, Oshitani N, Matsumoto T, Nishikawa H, Arakawa T. Prevalence of gastroesophageal reflux disease and gastroesophageal reflux disease symptoms in Japan. J Gastroenterol Hepatol 2005; 20(1):26-9.

23) Geisinger KR, Cassidy KT, Nardi R, Castello DO. The histologic development of acid-induced oesophagitis in the cat. Mod Pathol 1990; 3: 619-24.

24) Gelbmann CM, Schteingart CD, Thompson SM, Hofmann AF, Barrett KE. Mast cells and histamine contribute to bile acid-stimulated sectretion int he mouse colon. J Clin Invest 1995; 95: 2831-2839.

25) Ghyczy M, Torday C, Boros M. Simulatneous generation of methane, carbon dioxide and carbon monoxide from choline and ascorbic acid: a defensive mechanism against reductive stress? FASEB J 2003; 17: 1124-6.

26) Goh KL. Changing epidemiology of gastroesophageal reflux disease in the Asian-Pacific region: an overview. J Gastroenterol Hepatol 2004; 19 (Suppl3): S22-5.

27) Gores GJ, Miyoshi H, Botla R, Aguilar HI, Bronk SF. Induction ofthe mitochondrial permeability transition as a mechanism of liver injury during cholestasis: a potential role for mitochondrial proteases. Biochim Biophys Acta 1998; 1366: 167-75.

28) Groner W, Winkelman JW, Harris AG, Ince C, Bouma GJ, Messmer K, Nadeau RG. Orthogonal polarization spectral imaging: a new method for study of the microcirculation. Nat Med 1999; 5: 1209-12.

29) Hardcastle J, Hardcastle PT, Chapman J, Taylor CJ. Taurocholic acid-induced secretion in normal and cystic fibrosis mouse ileum. J Pharm Pharmacol 2001; 53: 711-719.

30) Helmer KS, West SD, Shipley GL, Chang L, Cui Y, Mailman D, Mercer DW. Gastric nitric oxide synthase expression during endotoxemia: implications in mucosal defense in rats. Gastroenterology 2002; 123: 173-86.

31) Hirota WK, Loughney TM, Lazas DJ et al. Specialized intestinal metaplasia, dysplasia and cancer of the esophagus and esophagogastric junction: prevalence and clinical data. Gastroenterology 1999; 116: 277-285.

32) Kanwar S, WallaceJL, Befus D, Kubes P. Nitric oxide synthesis inhibition increases epithelial permeability via mast cells. Am J Physiol 1994; 266: G222-G229.

33) Kauer WHK, Peters JH, DeMeester TR, Ireland AP, Bremner CG, Hagen JA. Mixed reflux of gastric and duodenal juices is more harmful to the esophagus than gastric juice alone. Ann Surg 1995; 222-533.

34) Kidd PM. Phosphatidylcholine, a superior protectant against liver damage. Altern Med Rev 1996; 1: 258-274.

35) Krahenbuhl S, Talos C, Fischer S, Reichen J. Toxicity of bile acids ont he electron transport chain of isolated rat liver mitochondria. Hepatology 1994; 19: 471-9.

36) Kuebler WM, Abels C, Schuerer L, Goetz AE. Measurement of neutrophil content in brain and lung tissue by a modified myeloperoxidase assay. Int J Microcirc 1996; 16: 89-97.

37) Kuehl FA, Jacob TA, GanleyOH, Ormond RE, Meisinger MAP. The identification of N-(2-hydroxyethyl)-palmitamide as a naturally occuring anti-inflammatory agent. J Am Chem Soc 1957; 79: 5577-5578.

38) Lamprech W, Trautschold I. Adenosine 5'-triphosphate. Determination with hexokinase and glucose 6-phosphate dehydrogenase. In: Bergmeyer HU, ed. Methods of Enzymatic Analysis. New York: Verlag Chemie, Weinheim and Academic Press, 1976; 4: 2101-2109.

39) Lanas A, Royo Y, Ortego J, Molina M, Sainz R. Experimental esophagitis induced by acid and pepsin in rabbits mimicking human reflux esophagitis. Gastroenterology 1999; 116: 97-107.

40) Lieber CS, Leo MA, Aleynik SI, Aleynik MK, DeCarli LM. Polyenylphosphatidylcholine decreases alcohol-induced oxidative stress int he baboon. Alcohol Clin Exp Res 1997; 21: 375-379.

41) Lowry OH, Rosenbrough NJ, Farr AL, Randall Rj. Protein measurement with the phenol reagent. J Biol Chem 1951; 193: 265-75.

42) Martin GP, Marriott C. Membrane damage by bile salts: the protective function of phospholipids. J Pharm Pharmacol 1981; 33: 754-759.

43) McKie LD, Dunkin BJ, Pennanen MF, Dunlap KW, Harmon JW, Bass BL. Esophageal mucosal blood flow: a central role for calcitonin gene-related peptide. Surgery 1994; 116: 409-17.

44) McKie LD, Bass BL, Dunkin BJ, Harmon JW. Nitric oxide regulates basal but not capsaicin-, CGRP-, or bile-salt stimulated rabbit esophageal mucosal blood flow. Ann Surg 1995; 222: 186-92.

45) Miwa K, Sahara H, Segawa M, Kinami S, Sato T, Miyazaki I, Hattori T. Reflux of duodenal or gastro-duodenal contents induces esophageal carcinoma in rats. Int J Cancer 1996; 67: 269-74.

46) Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, patophysiology, and pharmacology. Pharmacol Rev 1991; 43: 109-42.

47) Quist RG, Ton-Nu HT, Lillienau J, Hoffmann AF, Barrett KE. Activation of mast cells by bile acids. Gastroenterology 1991; 101: 446-456.

48) Qu XW, Wang H, De Plaen IG, Rozenfeld RA, Hsueh W. Neuronal nitric oxide synthase (NOS) regulates the expression of inducible NOS in rat small intestine via modulation of nuclear kappa B. FASEB J 2001; 15: 439-446.

49) Qu XW, Wang H, Rozenfeld RA, Huang W, Hsueh W. Type I nitric oxide synthase (NOS) is the predominant NOS in rat small intestine. Regulation by platelet-activating factor. Biochim Biophys Acta 1999; 1451: 211-217.

50) Rees WD, Rhodes J, Williams BC, Owen E, Newcombe RG. Effect of mast cell degranulation on gastric mucosal damage produced by sodium taurocholate in the rat. Gastroenterology 1978; 74: 492-495.

51) Richter JE. Duodenogastric Reflux-induced (Alkaline) Esophagitis. Curr Treat Options Gastroenterol 2004; 7(1): 53-58.

52) Rockey DC, Chung JJ. Reuced nitric oxide production by endothelial cells in cirrhotic rat liver: endothelial dysfunction in portal hypertension. Gastroenterology 1998; 114: 344-51.

53) Rosser BG, Gores GJ. Liver cell necrosis: Cellular mechanisms and clinical implications. Gastroenterology 1995; 108: 252-75.

54) Salo JA, Kivilaakso E. Role of bile salts and trypsin in the pathogenesis of experimental alkaline esophagitis. Surgery 1983; 93: 525-532.

55) Salvemini D, Maisini E, Pistelli A, Mannaioni PF, Vane JR. Nitric oxide: a regulatory mediator of mast cell reactivity. J Cardiovasc Pharmacol 1991; 17: S258-S264.

56) Salzman AL. Nitric oxide int he gut. New Horiz 1995; 3: 352-64.

57) Sandler AD, Schmidt C, Richardson K, Murray J, Maher JW. Regulation of distal esophageal mucosal blood flow: the roles of nitric oxide and substance P. Surgery 1993; 114: 285-293.

58) Satoh A, Shimosegawa T, Kimura K, Moriizumi S, Masamune A, Koizumi M, Toyota T. Nitric oxide is overproduced by peritoneal macrophages in rat taurocholate pancreatitis: the mechanism of inducible nitric oxide synthase expression. Pancreas 1998, 17(4): 402-11.

59) Schmucker DL, Ohta M, Kanasi S, Sato Y, Kitani K. Hepatic injury induced by bile salts: correlation between biochemical and morphological events. Hepatology 1990; 12: 1216-1221.

60) Spievy JR, Bronk SF, Gores GJ. Glycochenodeoxycholate-induced lethal hepatocellular injury in rat hepatocytes. J Clin Invest 1993; 92: 17-24.

61) Southan GJ, Szabo C. Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. Biochem Pharmacol 1996; 51: 383-394.

62) Szabó A, Boros M, Kaszaki J, Nagy S. The role of mast cells in mucosal permeability changes during ischemia-reperfusion injury of the small intestine. Shock 1997; 8: 284-291.

63) Szabó C, Mitchell JA, Thiemermann C, Vane JR. Nitric oxide-mediated hyporeactivity to noradrenaline precedes the induction of nitric oxide synthase in endotoxin shock. Br J Pharmacol 1993; 108: 786-92.

64) Szentpáli K, Kaszaki J, Tiszlavicz L, Lázár G, Balogh Á, Boros M. Bile-induced adenosine triphophate depletion and mucosal damage during reflux esophagitis. Scand J Gastroenterol 2001; 36: 459-466.

65) Szentpáli K, Paszt A, Lázár G, Széll M, Kemény L, Dobozy A, Kaszaki J, Tiszlavicz L, Iván L, Boros M. Esophageal ATP synthase and keratinocyte growth factor gene expression changes after acid and bile-induced mucosal damage. Inflamm. Res. 2005; 54: 152-157.

66) Vaezi MF, Richter JE. Duodenogastroesophageal reflux and methods to monitor nonacidic reflux. Am J Med 2001; 111 Suppl 8A: 160S-8S.

67) Vaezi MF, Richter JE. Role of acid and duodenoesophageal reflux in gastroesophageal reflux disease. Gastroenterology 1996; 111: 1192-9.

68) Wei C, Khoo H, Lee K, Hon W. Differential expression and localization of nitric oxide syntheses in cirrhotic livers of bile duct-ligated rats. Nitric Oxide 2002; 7: 91-102.

69) Yan JJ, Jung Js, Lee J, Huh SO, Kim HS, Jung KC, Cho JY, Nam JS, Suh HW, Kim YH, Song DK. Therapeutic effects of lysophosphatidylcholine in experimental sepsis, Nat Med 2004; 10: 161-167.

70) Zeisel SH, Da Costa K, Franklin PD et al. Choline, an essential nutrient for humans.FASEB 1991; 5: 2093-2098.

71) Zhang XJ, Thompson JH, Mannick EE, Correa P, Miller MJ. Localization of inducible nitric oxide synthase mRNA in inflamed gastrointestinal mucosa by in situ reverse transcriptase-polymerase chain reaction. Nitric Oxide 1998; 2: 187-92.

72) Zimmermann H, Kurzen P, Klossner W, Renner EL, Marti U. Decreased constitutive hepatic nitric oxide synthase expression in secondary biliary fibrosis and its changes after Roux-en-Y choledocho-jejunostomy in the rat. J Hepatol 1996; 25: 567-73.

ANNEX